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(71) Applicant: GENENCOR INTERNATIONAL, INC. [US/US]; 1870 South Winton Road, 4 Cambridge Place, Rochester, NY 14618 (US).

(72) Inventor: FOWLER, Timothy; 1979 Eaton Avenue, San Carlos, CA 94070 (US).

(74) Agent: FARIS, Susan, K.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304–1013 (US).

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(54) Title: EGIII–LIKE CELLULASE COMPOSITIONS, DNA ENCODING SUCH EGIII COMPOSITIONS AND METHODS FOR OBTAINING SAME

(57) Abstract

The present invention relates to variant EGIII–like cellulases which have improved surfactant stability. The variant cellulases have surfactant sensitive residues replaced to a residue having improved stability.

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EGIII-LIKE CELLULASE COMPOSITIONS, DNA ENCODING SUCH EGIII COMPOSITIONS AND METHODS FOR OBTAINING SAME

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is directed to novel mutant cellulase compositions which have improved resistance to stress such as temperature and surfactant, which stresses known to be problematic when used in conjunction with cellulase. More specifically, the present invention relates to a family of mutant cellulase enzymes from fungi and bacteria which are related to EGIII produced by *Trichoderma reesei*, but which have certain mutations which provide resistance to, for example, temperature stress and the presence of surfactant compounds such as linear alkyl sulfonates.

2. State of the Art

Cellulases are enzymes which are capable of hydrolysis of the β-D-glucosidic linkages in celluloses. Cellulolytic enzymes have been traditionally divided into three major classes: endoglucanases, exoglucanases or cellobiohydrolases and β-glucosidases (Knowles, J. et al., (1987), *TIBTECH* 5, 255-261); and are known to be produced by a large number of bacteria, yeasts and fungi.

Primary among the applications that have been developed for the use of cellulolytic enzymes are those involving degrading (wood)cellulose pulp into sugars for (bio)ethanol production, textile treatments like 'stone washing' and 'biopolishing', and in detergent compositions. Thus, cellulases are known to be useful in the treatment of mechanical pulp (see e.g., PCT Publication No. WO 92/16687). Additionally, cellulases are known to be useful as a feed additive (see e.g., PCT Publication No. WO 91/04673) and in grain wet milling.

Of primary importance, however, cellulases are used in the treatment of textiles, i.e., in detergent compositions for assisting in the removal of dirt or grayish cast (see e.g., Great Britain Application Nos. 2,075,028, 2,095,275 and 2,094,826 which illustrate improved cleaning performance when detergents incorporate cellulase) or in the treatment of textiles prior to sale to improve the feel and appearance of the textile. Thus, Great Britain Application No. 1,358,599 illustrates

the use of cellulase in detergents to reduce the harshness of cotton containing fabrics and cellulases are used in the treatment of textiles to recondition used fabrics by making their colors more vibrant (see e.g., The Shizuoka Prefectural Hammamatsu Textile Industrial Research Institute Report, Vol. 24, pp. 54-61 (1986)). For example, repeated washing of cotton containing fabrics results in a grayish cast to the fabric which is believed to be due to disrupted and disordered fibrils, sometimes called "pills", caused by mechanical action. This greyish cast is particularly noticeable on colored fabrics. As a consequence, the ability of cellulase to remove the disordered top layer of the fiber and thus improve the overall appearance of the fabric has been of value.

Thus, cellulases have been shown to be effective in many industrial processes. Accordingly, there has been a trend in the field to search for specific cellulase compositions or components which have particularly effective performance profiles with respect to one or more specific applications. In this light, cellulases produced (expressed) in fungi and bacteria have been subject of attention. For example, cellulase produced by certain fungi such as *Trichoderma spp.* (especially *Trichoderma longibrachiatum*) have been given much attention because a complete cellulase system capable of degrading crystalline forms of cellulose is readily produced in large quantities via fermentation procedures. This specific cellulase complex has been extensively analyzed to determine the nature of its specific components and the ability of those components to perform in industrial processes. For example, Wood et al., "Methods in Enzymology", 160, 25, pages 234 et seq. (1988), disclose that complete fungal cellulase systems comprise several different enzyme classifications including those identified as exo-cellobiohydrolases (EC 3.2.1.91) ("CBH"), endoglucanases (EC 3.2.1.4) ("EG"), and ß-glucosidases (EC 3.2.1.21) ("BG"). The fungal cellulase classifications of CBH, EG and BG can be further expanded to include multiple components within each classification. U.S. Patent No. 5,475,101 (Ward et al.) discloses the purification and molecular cloning of one particularly useful enzyme called EGIII which is derived from *Trichoderma longibrachiatum*.

PCT Publication No. WO 94/14953 discloses endoglucanases which are encoded by a nucleic acid which comprises any one of a series of DNA sequences, each having 20 nucleotides.

Ooi et al., *Curr. Genet.*, Vol. 18, pp. 217-222 (1990) disclose the cDNA sequence coding for endoglucanase F1-CMC produced by *Aspergillus aculeatus*

which contains the amino acid strings NNLWG, ELMIW and GTEPFT. Sakamoto et al., *Curr. Genet.*, Vol. 27, pp. 435-439 (1995) discloses the cCNA sequence encoding the endoglucanase CMCase-1 From *Aspergillus kawachii* IFO 4308 which contains the amino acid strings ELMIW and GTEPFT. Ward et al., discloses the sequence of EGIII having the amino acid strings NNLWG, ELMIW and GTEPFT. Additionally, two cellulase sequences, one from *Erwinia carotovara* and *Rhodothermus marinus* are disclosed in Saarilahti et al., *Gene*, Vol. 90, pp. 9-14 (1990) and Hreggvidsson et al., *Appl. Environ. Microb.*, Vol. 62, No. 8, pp. 3047-3049 (1996) which contain the amino acid string ELMIW. However, none of these references discloses or suggests that these amino acid strings have any particular relevance in identifying or isolating other cellulases, and particularly fail to suggest that such cellulases are obtainable from such diverse organisms as bacteria, Actinomycetes and other filamentous fungi.

Despite knowledge in the art related to many cellulase compositions having applications in some or all of the above areas, there is a continued need for new cellulase compositions which have resistance to certain surfactant compositions generally present in compositions with which cellulases are generally used, i.e., household detergents, stonewashing compositions or laundry detergents. One problem with the prior art cellulases has been the sensitivity of such surfactant compositions, for example to linear alkyl sulfonates (LAS). Because surfactants are ubiquitous in detergents, the susceptibility of cellulases to inactivation from such compounds can be highly disadvantageous to their value in these detergents.

EGIII from *Trichoderma reesei* has very good resistance to LAS type compounds in comparison with many cellulases. Thus, it is useful to compare EGIII to such enzymes with the intent of improving their performance in the presence of surfactant.

SUMMARY OF THE INVENTION

It is an object of the invention to provide for novel variant EGIII-like cellulase compositions which have improved performance in the presence of surfactants.

It is a further object of the invention to provide for novel mutant EGIII-like cellulase compositions which have improved performance under conditions of thermal stress.

It is a further object of the invention to provide for novel variant EGIII-like cellulase containing compositions which will provide excellent performance in detergent applications, including laundry detergents.

It is a further object of the invention to provide for novel variant EGIII-like cellulase containing compositions which have improved performance attributes for use in the textiles treatment field.

It is a further object of the invention to provide for novel variant EGIII-like cellulase composition which have improved characteristics for the reduction of biomass, as an additive in animal feed, in starch processing and in baking applications.

According to the present invention, a variant EGIII-like cellulase is provided wherein one or more amino acids are modified or deleted to confer improved performance, including stability in the presence of thermal and/or surfactant mediated stress. Preferably, the amino acids to be modified corresponds in position to residues 11, 12, 23, 27, 32, 51, 55, 57, 79, 81, 93, 107, 159, 179, 183 and/or 204 in EGIII from *Trichoderma reesei* except that the residue is not the specific amino acid as present at that position in EGIII. Preferably the modifications correspond to L11, I12, W23, T27, T32, A51, S55, G57, S79, A81, S93, N107, S159, T179, N183 and/or A204 or a conservative substitution thereof.

In a preferred embodiment of the present invention, the variant EGIII-like cellulase is an endoglucanase. Also preferably, the enzyme is derived from a fungal or bacterial source, most preferably from a filamentous fungus.

In another embodiment of the present invention, a DNA encoding the variant EGIII-like cellulase according to the invention is provided. Also provided are expression vectors comprising that DNA, host cells transformed with such expression vectors and variant EGIII-like cellulases produced by such host cells.

As shown in more detail below, the substitutions identified herein are important to the performance of EGIII-like enzymes in the presence of surfactant, e.g., detergents, and also in the presence of temperature stress. Accordingly, it is within the scope of the present invention to use of the EGIII-like enzyme in textile treatment, e.g., in laundry detergent or stonewashing compositions, in the reduction of biomass, in the production of feed additives or treatment of feed, in the treatment of wood pulp for the production of paper or pulp based products, and in the treatment of starch during grain wet milling or dry milling to facilitate the production of glucose, high fructose corn syrup and/or alcohol.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the amino acid sequence of EGIII from *Trichoderma longibrachiatum*.

Fig. 2 illustrates the DNA sequence of EGIII from *Trichoderma longibrachiatum* without introns.

Fig. 3 illustrates an alignment of the full length sequence of 20 EGIII-like cellulases in alignment with EGIII, indicating equivalent residues based on primary sequence modeling, including those derived from *Trichoderma reesei, Hypocrea schweinitzii, Aspergillus aculeatus, Aspergillus kawachii (1), Aspergillus kawachii (2), Aspergillus oryzae, Humicola grisea, Humicola insolens, Chaetomium brasilliense, Fusarium equiseti, Fusarium javanicum (1), Fusarium javanicum (2), Gliocladium roseum (1), Gliocladium roseum (2), Gliocladium roseum (3), Gliocladium roseum (4), Memnoniella echinata, Emericella desertoru, Actinomycete 11AG8, Streptomyces lividans CelB, Rhodothermus marinus*, and *Erwinia carotovara*.

Fig. 4 illustrates a comparison of the depilling performance of EGIII, an EGIII-like cellulase from *Hypocrea schweinitzii*, and a combination of EGIII and an EGIII-like cellulase from *Hypocrea schweinitzii* in LAS containing detergent at 40°C.

DETAILED DESCRIPTION OF THE INVENTION

Applicants have isolated novel members of a family of cellulases which have homology to EGIII from *Trichoderma reesei*. Analysis of these cellulases has resulted in differential performance between the cellulases, despite significant homology. In particular, it was discovered that the EGIII-like cellulase from *Hypocrea schweinitzii* has significantly diminished performance under conditions of thermal stress and/or in the presence of surfactants. This discovery is particularly pertinent as EGIII differs from its *Hypocrea schweinitzii* relative in only 14 positions indicating that these 14 positions have a significant impact on the stability and/or performance of EGIII. Thus, Applicants discovered that by optimizing in an EGIII-like enzyme the 14 residues which differ between EGIII and the EGIII-like cellulase from *Hypocrea schweinitzii*, it should be possible to confer significant performance benefits on the EGIII-like cellulase.

Accordingly, the present invention relates to a variant EGIII-like cellulase having improved stability to surfactants which is obtained from organisms other

than *Trichoderma reesei*. The variant is characterized by having one or more surfactant sensitive residues replaced with a residue conferring improved surfactant stability at that site. Preferably, the surfactant sensitive residue is replaced with the residue present in EGIII at the equivalent position or a conservative substitution thereto. Also preferably, the variant EGIII-like cellulase comprises a modification at a position selected from the group consisting of one or more of the residues corresponding to residues 11, 12, 23, 27, 32, 51, 55, 57, 79, 81, 93, 107, 159, 179, 183 and/or 204 in EGIII. Most preferably, the modification is selected from the group consisting of one or more of the following: a leucine at the position corresponding to residue 11 in EGIII (L11), an isoleucine at the position corresponding to residue 12 in EGIII (I12), a tryptophan at the position corresponding to residue 23 in EGIII (W23), a threonine at the position corresponding to residue 27 in EGIII (T27), a threonine at the position corresponding to residue 32 in EGIII (T32), an alanine at the position corresponding to residue 51 in EGIII (A51), a serine at the position corresponding to residue 55 in EGIII (S55), a glycine at the position corresponding to residue 57 in EGIII (G57), a serine at the position corresponding to residue 79 in EGIII (S79), an alanine at the position corresponding to residue 81 in EGIII (A81), a serine at the position corresponding to residue 93 in EGIII (S93), an asparagine at the position corresponding to residue 107 in EGIII (N107), a serine at the position corresponding to residue 159 in EGIII (S159), a threonine at the position corresponding to residue 179 in EGIII (T179), a asparagine at the position corresponding to residue 183 in EGIII (N183) and/or an alanine at the position corresponding to residue 204 in EGIII (A204). Conservative substitutions of the above may also be inserted in the EGIII-like cellulase, for example, leucine may be isoleucine, isoleucine may be leucine, tryptophan may be tyrosine, threonine may be asparagine, alanine may be glycine, serine may be asparagine, glycine may be proline and asparagine may be threonine.

Within the specification, certain terms are disclosed which are defined below so as to clarify the nature of the claimed invention.

"Cellulase" is a well classified category of enzymes in the art and includes enzymes capable of hydrolyzing cellulose polymers to shorter cellooligosaccharide oligomers, cellobiose and/or glucose. Common examples of cellulase enzymes include exo-cellobiohydrolases and endoglucanases and are obtainable from many species of cellulolytic organisms, particularly including fungi and bacteria.

"EGIII" cellulase refers to the endoglucanase component described in Ward et al., U.S. Patent No. 5,475,101 and Proceedings on the SecondTRICEL Symposium on *Trichoderma Reesei* Cellulases And Other Hydrolases, Suominen & Reinikainen eds., Espoo Finland (1993), pp. 153-158 (Foundation for Biotechnical and Industrial Fermentation Research, Vol. 8). As discussed therein, EGIII is derived from *Trichoderma reesei (longibrachiatum)* and is characterized by a pH optimum of about 5.8, an isoelectric point (pI) of about 7.4 and a molecular weight of about 25 kD. The enzyme commonly referred to as EGII from *Trichoderma reesei* has been previously referred to in the literature by the nomenclature EGIII by some authors, but that enzyme differs substantially from the enzyme defined herein as EGIII in terms of molecular weight, pI and pH optimum.

"EG-III like enzyme", "EGIII-like protein" or "EGIII-like cellulase" according to the present invention means enzymes which are related to EGIII by having certain amino acid strings in common with EGIII. Thus and EGIII like cellulase comprises an enzyme having cellulolytic activity which comprises an amino acid sequence comprising therein an amino acid string selected from the group consisting of one or more of:

(a) Asn-Asn-(Leu/Phe/Lys/Ile)-Trp-Gly

(b) Glu-(Leu/Phe/Ile)-Met-Ile-Trp

(c) Gly-Thr-Glu-Pro-Phe-Thr;

(d) (Ser/Tyr/Cys/Trp/Thr/Asn/Lys/Arg)-(Val/Pro)-(Lys/Ala)-(Ser/Ala)-(Tyr/Phe); and

(e) Lys-Asn-Phe-Phe-Asn-Tyr.

In one embodiment, the enzyme of the invention further has significant structural and/or sequence homology to EGIII. Thus, in one aspect of this embodiment of the invention, the enzyme has at least 30%, preferably at least 40% and most preferably at least 60% amino acid identity to EGIII. However, it should be recognized that homology alone is often not an appropriate measure for whether a particular enzyme identified by the methods described herein represents an EGIII-like enzyme. Accordingly, while homologous enzymes are indeed detected by the methods described and exemplified herein, the degree of homology should not be seen as limiting the scope of the invention.

It is contemplated that the EGIII-like cellulases of the invention may be found in many organisms which produce cellulases. However, likely sources of EGIII-like cellulase include those derived from a bacterial or fungal sources, and more

particularly, from an Actinomycete, a Bacillus or a filamentous fungus. In a preferred embodiment, the cellulase is derived from the filamentous fungal family Metazoa, preferably Euascomycetes. Within Metazoa, fungal phylogenetic classifications which produce EGIII-like cellulases include the mitosporic Pyrenomycetes (including Acremonium), Sordariales (including Thielavia), Hypocreales (including Nectriaceae such as Fusarium, Necitia, Verticillium, Myrothecium and Gliocladium; and Hypocrea) and Eurotiales (including mitosporic Trichocomaceae such as Aspergillus and Penicillium).

The Euascomycete preferably belongs to Diaporthales, Halosphaeriales, Microascales, Ophiostomatales, Phyllachorales, Sordariales or Xylariales. Also preferably, the Eusacomycete belongs to Hypocreales comprising Clavicipitaceae, Melanosporaceae, Nectriaceae, Niessliaceae or Mitosporic Hypocreales. Further preferably, the Euascomycete belongs to Hypocreaceae, wherein said Hypocreaceae does not comprise Trichoderma. Most preferably, the Euascomycete is *Gliocladium spp., Fusarium spp., Acremonium spp., Myceliophtora spp., Verticillium spp., Myrothecium spp., Penicillium spp., Chaetomium spp., Emercella spp.,* and *Phanerochaete spp.* Specific organisms which are contemplated as possessing EGIII-like cellulases include *Chaetomium thermophilum var. therm., Chaetomium atrobrunneum, Chaetomium brasiliense, Chaetomium globosum, Chaetomium vitellium, Paecilomyces lilacinus, Chaetomium thermophilum var. dissitum, Humicola insolens, Humicola brevis, Memnoniella echinata, Fusarium equiseti, Fusarium oxysporum, fusarium stilboides, Myceliophthora thermophila, Fusarium javanicum, Humicola grisea var. thermoidea, Stibella thermophila, Melanocarpus albomyces, Arthrobotrys superba, Myceliophthora hinunilea, Chaetomium pachypodiodes, Myrothecium verrucaria, Penicillium crysogenum, Malbranchea sulfurea, Lunulospora curvula, Emericella desertorum, Acremonium strictum, Cylindrocarpon heteronema,* and *Ulocladium chartarum.* Within the Actinomycetes, *Streptomyces* appears to possess EGIII-like cellulases.

EGIII-like cellulases according to the invention may be obtained according to the following methods. DNA primers are constructed which encode an amino acid sequence selected from the group consisting of one or more of:

(a) Asn-Asn-(Leu/Phe/Lys/Ile)-Trp-Gly

(b) Glu-(Leu/Phe/Ile)-Met-Ile-Trp

(c) Gly-Thr-Glu-Pro-Phe-Thr;

(d) (Ser/Tyr/Cys/Trp/Thr/Asn/Lys/Arg)-(Val/Pro)-(Lys/Ala)-(Ser/Ala)-(Tyr/Phe); and

(e) Lys-Asn-Phe-Phe-Asn-Tyr.

and used to obtain DNA, and genes, encoding enzymes having cellulolytic activity according to established methods. In addition, the EGIII of the invention may be obtained by methods conventional in molecular biology, e.g., PCR cloning, using one of the cellulase backbones identified herein as an EGIII like cellulase.

In a preferred embodiment according to this aspect of the invention, degenerate primers are prepared corresponding to one or more of the above peptides. The peptides are combined with a genomic DNA from a target organism (i.e., the organism in which the EGIII-like cellulase is sought) under conditions suitable to initiate a standard PCR reaction. In this embodiment, it is advantageous to select degenerate primers corresponding to peptides (a) and/or (d) plus primers corresponding to (c) and/or (e) and perform PCR with those peptides. After the PCR reaction has been performed, the resulting DNA is run on a polyacrylamide gel and bands corresponding in size to the EGIII fragment comprising peptides (a) and/or (d) in addition to (c) and/or (e), i.e., those in the 400-1000 base pair range, are selected out. These fragments are pooled and reamplified using primers corresponding to peptides (a) and/or (d) plus primers corresponding to peptide (b) or, alternatively, using primers corresponding to peptide (c) and/or (e) plus primers corresponding to peptide (b). Strong bands of the expected size (in the case of EGIII-like cellulases, the bands will correspond to the approximately 250-500 base pair range) are excised and sequenced. The sequence is then used to design exact match primers and these primers used with the technique referred to as rapid amplification of genomic DNA ends to obtain the full length gene, see e.g., Mizobuchi et al., *BioTechniques*, Vol. 15, No. 2, pp. 215-216 (1993).

However, it is also possible to use the degenerate DNA's as hybridization probes against a genomic library obtained from a target organism to analyze whether a given fragment correlates to a similar sequence in the target organism. A useful hybridization assay is as follows: Genomic DNA from a particular target source is fragmented by digestion with a restriction enzyme(s), e.g., EcoR I, Hind III, Bam HI, Cla I, Kpn I, Mlu I, Spe I, Bgl II, Nco I, Xba I, Xho I and Xma I (supplied by New England Biolabs, Inc., Beverly, MA and Boehringer Mannheim) according to the manufacturer's instructions. The samples are then electrophoresed through an agarose gel (such as, for example, 0.7% agarose) so that separation of DNA

fragments can be visualized by size. The gel may be briefly rinsed in distilled H_2O and subsequently depurinated in an appropriate solution (such as, for example, 0.25M HCl) with gentle shaking followed by denaturation for 30 minutes (in, for example, 0.4 M NaOH). A renaturation step may be included in which the gel is placed in 1.5 M NaCl, IM Tris, pH 7.0 with gentle shaking for 30 minutes. The DNA should then be transferred onto an appropriate positively charged membrane, for example the *Maximum Strength Nytran Plus* membrane (Schleicher & Schuell, Keene, N.H.), using a transfer solution (such as, for example, 6XSSC (900 mM NaCl, 90 mM trisodium citrate). After the transfer is complete, generally at about 2 hours or greater, the membrane is rinsed and air dried at room temperature after using a rinse solution (such as, for example, 2X SSC[2X SSC = 300 mM NaCl, 30 mM trisodium citrate]). The membrane should then be prehybridized, (for approximately 2 hours or more) in a suitable prehybridization solution (such as, for example, an aqueous solution containing per 100 mls: 30-50 mls formamide, 25 mls of 20X SSPE (1X SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.7), 2.5 mls of 20% SDS, 1 ml of 10 mg/ml sheared herring sperm DNA).

A DNA probe corresponding to the peptide sequences above should be isolated by electrophoresis in an agarose gel, the fragment excised from the gel and recovered from the excised agarose. This purified fragment of DNA is then labeled (using, for example, the *Megaprime* labeling system according to the instructions of the manufacturer to incorporate P^{32} in the DNA (Amersham International plc, Buckinghamshire, England)). The labeled probe is denatured by heating to 95° C for 5 minutes and immediately added to the prehybridization solution above containing the membrane. The hybridization reaction should proceed for an appropriate time and under appropriate conditions, for example, for 18 hours at 37°C with gentle shaking. The membrane is rinsed (for example, in 2X SSC/0.3% SDS) and then washed with an appropriate wash solution and with gentle agitation. The stringency desired will be a reflection of the conditions under which the membrane (filter) is washed.

Specifically, the stringency of a given reaction (i.e., the degree of homology necessary for successful hybridization) will largely depend on the washing conditions to which the filter from the Southern Blot is subjected after hybridization. "Low-stringency" conditions as defined herein will comprise washing a filter from a Southern Blot with a solution of 0.2X SSC/0.1% SDS at 20° C for 15 minutes. Standard-stringency conditions comprise a further washing step comprising washing

the filter from the Southern Blot a second time with a solution of 0.2X SSC/0.1% SDS at 37°C for 30 minutes.

The DNA which hybridizes with the DNA primers outlined above and thus identified by this method a corresponding EGIII encoding gene may be isolated by routine methods and used to express the corresponding EGIII-like cellulase according to routine techniques. A preferred cloning procedure comprises the rapid amplification of genomic DNA ends described in, e.g., Mizobuchi et al., BioTechniques, Vol. 15, No. 2, pp. 215-216 (1993). Upon obtaining the cloned gene, routine methods for insertion of the DNA into a vector which can then be transformed into a suitable host cell are used. Culturing the transformed host cell under appropriate conditions then results in production of the EGIII-like cellulase which can be obtained, purified and prepared as necessary for a particular application.

The EGIII-like cellulases of the invention are preferably isolated or purified. In the context of the present invention, purification or isolation generally means that the EGIII-like cellulase is altered from its natural state by virtue of separating the EGIII-like cellulase from some or all of the naturally occurring substituents with which it is associated in nature, e.g., the source organism or other cellulases or enzymes expressed by the source organism in conjunction with the EGIII cellulase. Similarly, the EGIII-like cellulases of the invention may be combined with other components which are not naturally present in the natural state. Isolation of purification may be accomplished by art recognized separation techniques such as ion exchange chromatography, affinity chromatography, hydrophobic separation, dialysis, protease treatment, ammonium sulphate precipitation or other protein salt precipitation techniques, centrifugation, size exclusion chromatography, filtration, microfiltration, gel electrophoresis or separation on a gradient to remove whole cells, cell debris, impurities, extraneous proteins, or enzymes undesired in the final composition.

A residue in an EGIII-like cellulase which is "corresponding" or "equivalent" to a residue present in EGIII means a residue which exists in an equivalent position to that in EGIII, as indicated by primary sequence homology, tertiary structural homology (as shown by, i.e., crystal structure or computer modeling) or functional equivalence. A variant EGIII-like cellulase has an amino acid sequence which is derived from the amino acid sequence of a precursor EGIII-like cellulase. The precursor cellulases include naturally occurring cellulases and recombinant

cellulases (as defined herein). The amino acid sequence of the EGIII-like cellulase variant is derived from the precursor EGIII-like cellulase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the precursor DNA sequence which encodes the amino acid sequence of the precursor cellulase rather than manipulation of the precursor cellulase enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in commonly owned US patent 4,760,025 and 5,185,258. Specific residues corresponding to the positions which are responsible for instability in the presence of surfactant are identified herein for substitution or deletion. The amino acid position number (i.e., +11) refers to the number assigned to the mature *Trichoderma reesei* EGIII sequence presented in Fig. 1. The invention is directed to the mutation of EGIII-like cellulases which contain amino acid residues at positions which are equivalent to the particular identified residue in *Trichoderma reesei* EGIII. A residue (amino acid) of a precursor cellulase is equivalent to a residue of *Trichoderma reesei* EGIII if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or is functionally analogous to a specific residue or portion of that residue in *Trichoderma reesei* EGIII (i.e., having the same or similar functional capacity to combine, react, or interact chemically or structurally).

"Surfactant" means any compound generally recognized in the art as having surface active qualities. Thus, for example, surfactants comprise anionic, cationic and nonionic surfactants such as those commonly found in detergents. Cationic surfactants and long-chain fatty acid salts include saturated or unsaturated fatty acid salts, alkyl or alkenyl ether carboxylic acid salts, α-sulfofatty acid salts or esters, amino acid-type surfactants, phosphate ester surfactants, quaternary ammonium salts including those having 3 to 4 alkyl substituents and up to 1 phenyl substituted alkyl substituents. Examples of cationic surfactants and long-chain fatty acid salts are disclosed in British Patent Application No. 2 094 826 A, the disclosure of which is incorporated herein by reference. The composition may contain from about 1 to about 20 weight percent of such cationic surfactants and long-chain fatty acid salts. Anionic surfactants include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; and alkanesulfonates. Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth metal ions such as calcium and

magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3. Ampholytic surfactants include quaternary ammonium salt sulfonates, and betaine-type ampholytic surfactants. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule. Nonionic surfactants may comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, fatty acid glycerine monoesters, and the like. Examples of surfactants for use in this invention are disclosed in British Patent Application No. 2 094 826 A, the disclosure of which is incorporated herein by reference. Mixtures of such surfactants can also be used.

"Cellulose containing fabric" means any sewn or unsewn fabrics, yarns or fibers made of cotton or non-cotton containing cellulose or cotton or non-cotton containing cellulose blends including natural cellulosics and manmade cellulosics (such as jute, flax, ramie, rayon, and lyocell). Included under the heading of manmade cellulose containing fabrics are regenerated fabrics that are well known in the art such as rayon. Other manmade cellulose containing fabrics include chemically modified cellulose fibers (e.g, cellulose derivatized by acetate) and solvent-spun cellulose fibers (e.g. lyocell). Specifically included within the definition of cellulose containing fabric is any yarn or fiber made of such materials. Cellulose containing materials are often incorporated into blends with materials such as synthetic fibers and natural non-cellulosic fibers such as wool and silk.

"Cotton-containing fabric" means sewn or unsewn fabrics, yarns or fibers made of pure cotton or cotton blends including cotton woven fabrics, cotton knits, cotton denims, cotton yarns, raw cotton and the like. When cotton blends are employed, the amount of cotton in the fabric is preferably at least about 35 percent by weight cotton. When employed as blends, the companion material employed in the fabric can include one or more non-cotton fibers including cellulosic or synthetic fibers such as polyamide fibers (for example, nylon 6 and nylon 66), acrylic fibers (for example, polyacrylonitrile fibers), and polyester fibers (for example, polyethylene terephthalate), polyvinyl alcohol fibers (for example, Vinylon), polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers and aramid fibers.

"Stonewashing composition" means a formulation for use in stonewashing cellulose containing fabrics. Stonewashing compositions are used to modify cellulose containing fabrics prior to presentation for consumer sale, i.e., during the

manufacturing process. In contrast, detergent compositions are intended for the cleaning of soiled garments.

"Stonewashing" means the treatment of cellulose containing fabric with a cellulase solution under agitating and cascading conditions, i.e., in a rotary drum washing machine, to impart a "stonewashed" appearance to the denim. The cellulase solution according to the instant invention will functionally replace the use of stones in such art recognized methods, either completely or partially. Methods for imparting a stonewashed appearance to denim are described in U.S. Patent No. 4,832,864 which is incorporated herein by reference in its entirety. Generally, stonewashing techniques have been applied to indigo dyed cotton denim.

"Detergent composition" means a mixture which is intended for use in a wash medium for the laundering of soiled cellulose containing fabrics. In the context of the present invention, such compositions may include, in addition to cellulases and surfactants, additional hydrolytic enzymes, builders, bleaching agents, bleach activators, bluing agents and fluorescent dyes, caking inhibitors, masking agents, cellulase activators, antioxidants, and solubilizers. Such compositions are generally used for cleaning soiled garments and are not used during the manufacturing process, in contrast to stonewashing compositions. Detergent compositions comprising cellulase are described in, for example, Clarkson et al., U.S. Patent No. 5,290,474 and EP Publication No. 271 004, incorporated herein by reference.

"Variant" means a protein which is derived from a precursor protein (e.g., the native protein) by addition of one or more amino acids to either or both the C- and N-terminal end, substitution of one or more amino acids at one or a number of different sites in the amino acid sequence, deletion of one or more amino acids at either or both ends of the protein or at one or more sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the amino acid sequence. The preparation of an enzyme variant is preferably achieved by modifying a DNA sequence which encodes for the native protein, transformation of that DNA sequence into a suitable host, and expression of the modified DNA sequence to form the derivative enzyme. The variant EGIII-like enzyme of the invention includes peptides comprising altered amino acid sequences in comparison with a precursor enzyme amino acid sequence wherein the variant EGIII-like enzyme retains the characteristic cellulolytic nature of the precursor enzyme but which may have altered properties in some specific aspect. For example, a variant

EGIII-like enzyme may have an increased pH optimum or increased temperature or oxidative stability but will retain cellulolytic activity. It is contemplated that the variants according to the present invention may be derived from a DNA fragment encoding a cellulase variant EGIII-like enzyme wherein the functional activity of the expressed cellulase derivative is retained. For example, a DNA fragment encoding a cellulase may further include a DNA sequence or portion thereof encoding a hinge or linker attached to the cellulase DNA sequence at either the 5' or 3' end wherein the functional activity of the encoded cellulase domain is retained.

"Expression vector" means a DNA construct comprising a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome-binding sites on the mRNA, and sequences which control termination of transcription and translation. Different cell types are preferably used with different expression vectors. A preferred promoter for vectors used in *Bacillus subtilis* is the AprE promoter; a preferred promoter used in *E. coli* is the Lac promoter, a preferred promoter used in *Saccharomyces cerevisiae* is *PGK1*, a preferred promoter used in *Aspergillus niger* is *glaA*, and a preferred promoter for *Trichoderma reesei* is *cbhI*. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, under suitable conditions, integrate into the genome itself. In the present specification, plasmid and vector are sometimes used interchangeably. However, the invention is intended to include other forms of expression vectors which serve equivalent functions and which are, or become, known in the art. Thus, a wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from *E. coli* including col E1, pCR1, pBR322, pMb9, pUC 19 and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs e.g., the numerous derivatives of phage λ, e.g., NM989, and other DNA phages, e.g., M13 and filamentous single stranded DNA phages, yeast plasmids such as the 2μ plasmid or derivatives thereof, vectors useful in eukaryotic cells, such as vectors useful in animal cells and vectors derived from combinations of

plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other expression control sequences. Expression techniques using the expression vectors of the present invention are known in the art and are described generally in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Press (1989). Often, such expression vectors including the DNA sequences of the invention are transformed into a unicellular host by direct insertion into the genome of a particular species through an integration event (see e.g., Bennett & Lasure, *More Gene Manipulations in Fungi*, Academic Press, San Diego, pp. 70-76 (1991) and articles cited therein describing targeted genomic insertion in fungal hosts, incorporated herein by reference).

"Host strain" or "host cell" means a suitable host for an expression vector comprising DNA according to the present invention. Host cells useful in the present invention are generally procaryotic or eucaryotic hosts, including any transformable microorganism in which expression can be achieved. Specifically, host strains may be *Bacillus subtilis, Escherichia coli, Trichoderma reesei, Saccharomyces cerevisiae* or *Aspergillus niger.* Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of both replicating vectors encoding the variant EGIII-like enzymes or expressing the desired peptide product. In a preferred embodiment according to the present invention, "host cell" means both the cells and protoplasts created from the cells of *Trichoderma sp.*

"Signal sequence" means a sequence of amino acids bound to the N-terminal portion of a protein which facilitates the secretion of the mature form of the protein outside of the cell. This definition of a signal sequence is a functional one. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

"DNA vector" means a nucleotide sequence which comprises one or more DNA fragments or DNA variant fragments encoding an EGIII-like cellulase or variants described above which can be used, upon transformation into an appropriate host cell, to cause expression of the variant EGIII-like cellulase.

"Functionally attached to" means that a regulatory region, such as a promoter, terminator, secretion signal or enhancer region is attached to a structural gene and controls the expression of that gene.

The present invention relates to the expression, purification and/or isolation and use of variant EGIII-like cellulases. These enzymes are preferably prepared by recombinant methods utilizing the gene identified and isolated according to the methods described above. However, enzymes for use in the present invention may be obtained by other art recognized means such as purification from natural isolates.

It is conceived by the inventors that the microorganism to be transformed for the purpose of expressing an EGIII-like cellulase according to the present invention may advantageously comprise a strain derived from *Trichoderma sp*. Thus, a preferred mode for preparing EGIII-like cellulases according to the present invention comprises transforming a *Trichoderma sp.* host cell with a DNA construct comprising at least a fragment of DNA encoding a portion or all of the EGIII-like cellulase detected as described above. The DNA construct will generally be functionally attached to a promoter. The transformed host cell is then grown under conditions so as to express the desired protein. Subsequently, the desired protein product is purified to substantial homogeneity.

However, it may in fact be that the best expression vehicle for a given DNA encoding a variant EGIII-like cellulase may differ. Thus, it may be that it will be most advantageous to express a protein in a transformation host which bears phylogenetic similarity to the source organism for the variant EGIII-like cellulase. Accordingly, the present description of a *Trichoderma spp.* expression system is provided for illustrative purposes only and as one option for expressing the variant EGIII-like cellulase of the invention. One of skill in the art, however, may be inclined to express the DNA encoding variant EGIII-like cellulase in a different host cell if appropriate and it should be understood that the source of the variant EGIII-like cellulase should be considered in determining the optimal expression host. Additionally, the skilled worker in the field will be capable of selecting the best expression system for a particular gene through routine techniques utilizing the tools available in the art.

In one embodiment, the strain comprises *T. reesei (longibrachiatum)* which is a useful strain for obtaining overexpressed protein. For example, RL-P37, described by Sheir-Neiss et al. in *Appl. Microbiol. Biotechnology*, 20 (1984) pp. 46-53 is known to secrete elevated amounts of cellulase enzymes. Functional equivalents of RL-P37 include *Trichoderma reesei (longibrachiatum)* strain RUT-

C30 (ATCC No. 56765) and strain QM9414 (ATCC No. 26921). It is contemplated that these strains would also be useful in overexpressing EGIII-like cellulases.

Where it is desired to obtain the EGIII-like cellulase in the absence of potentially detrimental native cellulolytic activity, it is useful to obtain a *Trichoderma* host cell strain which has had one or more cellulase genes deleted prior to introduction of a DNA construct or plasmid containing the DNA fragment encoding the EGIII-like cellulase. Such strains may be prepared by the method disclosed in U.S. Patent No. 5,246,853 and WO 92/06209, which disclosures are hereby incorporated by reference. By expressing an EGIII-like cellulase in a host microorganism that is missing one or more cellulase genes, the identification and subsequent purification procedures are simplified. Any gene from *Trichoderma sp.* which has been cloned can be deleted, for example, the *cbh1*, *cbh2*, *egl1*, and *egl3* genes as well as those encoding EGIII and/or EGV protein (see e.g., U.S. Patent No. 5,475,101 and WO 94/28117, respectively).

Gene deletion may be accomplished by inserting a form of the desired gene to be deleted or disrupted into a plasmid by methods known in the art. The deletion plasmid is then cut at an appropriate restriction enzyme site(s), internal to the desired gene coding region, and the gene coding sequence or part thereof replaced with a selectable marker. Flanking DNA sequences from the locus of the gene to be deleted or disrupted, preferably between about 0.5 to 2.0 kb, remain on either side of the selectable marker gene. An appropriate deletion plasmid will generally have unique restriction enzyme sites present therein to enable the fragment containing the deleted gene, including flanking DNA sequences, and the selectable marker gene to be removed as a single linear piece.

A selectable marker must be chosen so as to enable detection of the transformed fungus. Any selectable marker gene which is expressed in the selected microorganism will be suitable. For example, with *Trichoderma sp.*, the selectable marker is chosen so that the presence of the selectable marker in the transformants will not significantly affect the properties thereof. Such a selectable marker may be a gene which encodes an assayable product. For example, a functional copy of a *Trichoderma sp.* gene may be used which if lacking in the host strain results in the host strain displaying an auxotrophic phenotype.

In a preferred embodiment, a *pyr4*$^-$ derivative strain of *Trichoderma sp.* is transformed with a functional *pyr4* gene, which thus provides a selectable marker for transformation. A *pyr4*$^-$ derivative strain may be obtained by selection of

Trichoderma sp. strains which are resistant to fluoroorotic acid (FOA). The *pyr4* gene encodes orotidine-5'-monophosphate decarboxylase, an enzyme required for the biosynthesis of uridine. Strains with an intact *pyr4* gene grow in a medium lacking uridine but are sensitive to fluoroorotic acid. It is possible to select *pyr4⁻* derivative strains which lack a functional orotidine monophosphate decarboxylase enzyme and require uridine for growth by selecting for FOA resistance. Using the FOA selection technique it is also possible to obtain uridine requiring strains which lack a functional orotate pyrophosphoribosyl transferase. It is possible to transform these cells with a functional copy of the gene encoding this enzyme (Berges and Barreau, *Curr. Genet.* ,19, 1991, pp. 359-365). Selection of derivative strains is easily performed using the FOA resistance technique referred to above, and thus, the *pyr4* gene is preferably employed as a selectable marker.

To transform *pyr4⁻ Trichoderma sp.* so as to be lacking in the ability to express one or more cellulase genes, a single DNA fragment comprising a disrupted or deleted cellulase gene is then isolated from the deletion plasmid and used to transform an appropriate *pyr⁻ Trichoderma* host. Transformants are then identified and selected based on their ability to express the *pyr4* gene product and thus compliment the uridine auxotrophy of the host strain. Southern blot analysis is then carried out on the resultant transformants to identify and confirm a double crossover integration event which replaces part or all of the coding region of the genomic copy of the gene to be deleted with the *pyr4* selectable markers.

Although the specific plasmid vectors described above relate to preparation of *pyr⁻* transformants, the present invention is not limited to these vectors. Various genes can be deleted and replaced in the *Trichoderma sp.* strain using the above techniques. In addition, any available selectable markers can be used, as discussed above. In fact, any *Trichoderma sp.* gene which has been cloned, and thus identified, can be deleted from the genome using the above-described strategy.

As stated above, the host strains used are derivatives of *Trichoderma sp.* which lack or have a nonfunctional gene or genes corresponding to the selectable marker chosen. For example, if the selectable marker of *pyr4* is chosen, then a specific *pyr4⁻* derivative strain is used as a recipient in the transformation procedure. Similarly, selectable markers comprising *Trichoderma sp.* genes equivalent to the *Aspergillus nidulans* genes *amdS, argB, trpC, niaD* may be used. The

corresponding recipient strain must therefore be a derivative strain such as *argB*$^-$, *trpC*$^-$, *niaD*$^-$, respectively.

DNA encoding the EGIII-like cellulase is then prepared for insertion into an appropriate microorganism. According to the present invention, DNA encoding a EGIII-like cellulase comprises all of the DNA necessary to encode for a protein which has functional cellulolytic activity. The DNA fragment or DNA variant fragment encoding the EGIII-like cellulase or derivative may be functionally attached to a fungal promoter sequence, for example, the promoter of the *cbh1* or *egl1* gene.

It is also contemplated that more than one copy of DNA encoding a EGIII-like cellulase may be recombined into the strain to facilitate overexpression. The DNA encoding the EGIII-like cellulase may be prepared by the construction of an expression vector carrying the DNA encoding the cellulase. The expression vector carrying the inserted DNA fragment encoding the EGIII-like cellulase may be any vector which is capable of replicating autonomously in a given host organism or of integrating into the DNA of the host, typically a plasmid. In preferred embodiments two types of expression vectors for obtaining expression of genes are contemplated. The first contains DNA sequences in which the promoter, gene coding region, and terminator sequence all originate from the gene to be expressed. Gene truncation may be obtained where desired by deleting away undesired DNA sequences (e.g., coding for unwanted domains) to leave the domain to be expressed under control of its own transcriptional and translational regulatory sequences. A selectable marker is also contained on the vector allowing the selection for integration into the host of multiple copies of the novel gene sequences.

The second type of expression vector is preassembled and contains sequences required for high level transcription and a selectable marker. It is contemplated that the coding region for a gene or part thereof can be inserted into this general purpose expression vector such that it is under the transcriptional control of the expression cassettes promoter and terminator sequences. For example, pTEX is such a general purpose expression vector. Genes or part thereof can be inserted downstream of the strong *cbh*1 promoter.

In the vector, the DNA sequence encoding the EGIII-like cellulase of the present invention should be operably linked to transcriptional and translational sequences, i.e., a suitable promoter sequence and signal sequence in reading frame to the structural gene. The promoter may be any DNA sequence which

shows transcriptional activity in the host cell and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The signal peptide provides for extracellular production of the EGIII-like cellulase or derivatives thereof. The DNA encoding the signal sequence is preferably that which is naturally associated with the gene to be expressed, however the signal sequence from any suitable source, for example an exo-cellobiohydrolase or endoglucanase from *Trichoderma*, is contemplated in the present invention.

The procedures used to ligate the DNA sequences coding for the EGIII-like cellulase of the present invention with the promoter, and insertion into suitable vectors are well known in the art.

The DNA vector or construct described above may be introduced in the host cell in accordance with known techniques such as transformation, transfection, microinjection, microporation, biolistic bombardment and the like.

In the preferred transformation technique, it must be taken into account that the permeability of the cell wall to DNA in *Trichoderma sp.* is very low. Accordingly, uptake of the desired DNA sequence, gene or gene fragment is at best minimal. There are a number of methods to increase the permeability of the *Trichoderma sp.* cell wall in the derivative strain (i.e., lacking a functional gene corresponding to the used selectable marker) prior to the transformation process.

The preferred method in the present invention to prepare *Trichoderma sp.* for transformation involves the preparation of protoplasts from fungal mycelium. The mycelium can be obtained from germinated vegetative spores. The mycelium is treated with an enzyme which digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate and the like. Usually the concentration of these stabilizers varies between 0.8 M to 1.2 M. It is preferable to use about a 1.2 M solution of sorbitol in the suspension medium.

Uptake of the DNA into the host *Trichoderma sp.* strain is dependent upon the calcium ion concentration. Generally between about 10 mM $CaCl_2$ and 50 mM $CaCl_2$ is used in an uptake solution. Besides the need for the calcium ion in the uptake solution, other items generally included are a buffering system such as TE buffer (10 Mm Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropanesulfonic acid) and polyethylene glycol (PEG). It is believed that the polyethylene glycol acts to fuse the cell membranes thus permitting the contents

of the medium to be delivered into the cytoplasm of the *Trichoderma sp.* strain and the plasmid DNA is transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA tenderly integrated into the host chromosome.

Usually a suspension containing the *Trichoderma sp.* protoplasts or cells that have been subjected to a permeability treatment at a density of 10^8 to 10^9/ml, preferably 2 x 10^8/ml are used in transformation. A volume of 100 microliters of these protoplasts or cells in an appropriate solution (e.g., 1.2 M sorbitol; 50 mM $CaCl_2$) are mixed with the desired DNA. Generally a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. However, it is preferable to add about 0.25 volumes to the protoplast suspension. Additives such as dimethyl sulfoxide, heparin, spermidine, potassium chloride and the like may also be added to the uptake solution and aid in transformation.

Generally, the mixture is then incubated at approximately 0°C for a period of between 10 to 30 minutes. Additional PEG is then added to the mixture to further enhance the uptake of the desired gene or DNA sequence. The 25% PEG 4000 is generally added in volumes of 5 to 15 times the volume of the transformation mixture; however, greater and lesser volumes may be suitable. The 25% PEG 4000 is preferably about 10 times the volume of the transformation mixture. After the PEG is added, the transformation mixture is then incubated at room temperature before the addition of a sorbitol and $CaCl_2$ solution. The protoplast suspension is then further added to molten aliquots of a growth medium. This growth medium permits the growth of transformants only. Any growth medium can be used in the present invention that is suitable to grow the desired transformants. However, if *Pyr*$^+$ transformants are being selected it is preferable to use a growth medium that contains no uridine. The subsequent colonies are transferred and purified on a growth medium depleted of uridine.

At this stage, stable transformants may be distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium lacking uridine. Additionally, in some cases a further test of stability may made by growing the transformants on solid non-selective medium (i.e. containing uridine), harvesting spores from this culture medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium lacking uridine.

In a particular embodiment of the above method, the EGIII-like cellulases or derivatives thereof are recovered in active form from the host cell after growth in liquid media either as a result of the appropriate post translational processing of the novel EGIII-like cellulase or derivatives thereof.

The expressed EGIII-like cellulase may be recovered from the medium by conventional techniques including separations of the cells from the medium by centrifugation, filtration, and precipitation of the proteins in the supernatant or filtrate with a salt, for example, ammonium sulphate. Additionally, chromatography procedures such as ion exchange chromatography or affinity chromatography may be used. Antibodies (polyclonal or monoclonal) may be raised against the natural purified EGIII-like cellulase, or synthetic peptides may be prepared from portions of the EGIII-like cellulase molecule and used to raise polyclonal antibodies.

Treatment of textiles according to the present invention contemplates textile processing or cleaning with a composition comprising a cellulase. Such treating includes, but is not limited to, stonewashing, modifying the texture, feel and/or appearance of cellulose containing fabrics or other techniques used during manufacturing or cleaning/reconditioning of cellulose containing fabrics. Additionally, treating within the context of this invention contemplates the removal of "immature" or "dead" cotton, from cellulosic fabric or fibers. Immature cotton is significantly more amorphous than mature cotton and results in a lesser quality fabric when present due to, for example, uneven dyeing. The composition contemplated in the present invention further includes a cellulase component for use in washing of a soiled manufactured cellulose containing fabric. For example, the cellulase may be used in a detergent composition for washing laundry. Detergent compositions useful in accordance with the present invention include special formulations such as pre-wash, pre-soak and home-use color restoration compositions. Such treating compositions, as described herein, may be in the form of a concentrate which requires dilution or in the form of a dilute solution or form which can be applied directly to the cellulose containing fabric. General treatment techniques for cellulase treatment of textiles are described in, for example, EP Publication No. 220 016 and GB Application Nos. 1,368,599 and 2,095,275.

Treatment of a cellulosic material according to the present invention further contemplates the treatment of animal feed, pulp and/or paper, food and grain for purposes known in the art. For example, cellulase is known to increase the value of

animal feed, improve the drainability of wood pulp, enhance food products and reduce fiber in grain during the grain wet milling process or dry milling process.

Treating according to the instant invention comprises preparing an aqueous solution which contains an effective amount of cellulase together with other optional ingredients including, for example, a buffer, a surfactant, and/or a scouring agent. An effective amount of cellulase enzyme composition is a concentration of cellulase enzyme sufficient for its intended purpose. Thus, for example, an "effective amount" of cellulase in a stonewashing composition according to the present invention is that amount which will provide the desired effect, e.g., to produce a worn and faded look in the seams and on fabric panels. Similarly, an "effective amount" of cellulase in a composition intended for improving the feel and/or appearance of a cellulose containing fabric is that amount which will produce measurable improvements in the feel, e.g., improving the smoothness of the fabric, or appearance, e.g., removing pills and fibrils which tend to reduce the sharpness in appearance of a fabric. The amount of cellulase employed is also dependent on the equipment employed, the process parameters employed (the temperature of the cellulase treatment solution, the exposure time to the cellulase solution, and the like), and the cellulase activity (e.g., a particular solution will require a lower concentration of cellulase where a more active cellulase composition is used as compared to a less active cellulase composition). The exact concentration of cellulase in the aqueous treatment solution to which the fabric to be treated is added can be readily determined by the skilled artisan based on the above factors as well as the desired result. In stonewashing processes, it has generally been preferred that the cellulase be present in the aqueous treating solution in a concentration of from about 0.5 to 5,000 ppm and most preferably about 10 to 200 ppm total protein. In compositions for the improvement of feel and/or appearance of a cellulose containing fabric, it has generally been preferred that the cellulase be present in the aqueous treating solution in a concentration of from about 0.1 to 2000 ppm and most preferably about 0.5 to 200 ppm total protein.

In a preferred treating embodiment, a buffer is employed in the treating composition such that the concentration of buffer is sufficient to maintain the pH of the solution within the range wherein the employed cellulase exhibits activity which, in turn, depends on the nature of the cellulase employed. The exact concentration of buffer employed will depend on several factors which the skilled artisan can readily take into account. For example, in a preferred embodiment, the buffer as

well as the buffer concentration are selected so as to maintain the pH of the final cellulase solution within the pH range required for optimal cellulase activity. The determination of the optimal pH range of the cellulases of the invention can be ascertained according to well known techniques. Suitable buffers at pH within the activity range of the cellulase are well known to those skilled in the art in the field.

In addition to cellulase and a buffer, the treating composition may optionally contain a surfactant. Suitable surfactants include any surfactant compatible with the cellulase and the fabric including, for example, anionic, non-ionic and ampholytic surfactants. Suitable anionic surfactants for use herein include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; alkanesulfonates and the like. Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth metal ions such as calcium and magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3. Ampholytic surfactants include quaternary ammonium salt sulfonates, and betaine-type ampholytic surfactants. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule. Nonionic surfactants generally comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, and fatty acid glycerine monoesters. Mixtures of surfactants can also be employed in manners known to those skilled in the art.

A concentrated cellulase composition can be prepared for use in the methods described herein. Such concentrates contain concentrated amounts of the cellulase composition described above, buffer and surfactant, preferably in an aqueous solution. When so formulated, the cellulase concentrate can readily be diluted with water so as to quickly and accurately prepare cellulase preparations having the requisite concentration of each constituent. When aqueous concentrates are formulated, these concentrates can be diluted so as to arrive at the requisite concentration of the components in the cellulase solution as indicated above. As is readily apparent, such cellulase concentrates will permit facile formulation of the cellulase solutions as well as permit feasible transportation of the composition to the location where it will be used. The treating concentrate can be in any art recognized form, for example, liquid, emulsion, gel, or paste. Such forms are well known to those skilled in the art.

When a solid cellulase concentrate is employed, the cellulase composition may be a granule, a powder, an agglomerate or a solid disk. The granules can be formulated so as to contain materials to reduce the rate of dissolution of the granules into the wash medium. Such materials and granules are disclosed in U.S. Patent No. 5,254,283 which is incorporated herein by reference in its entirety.

Other materials can also be used with or placed in the cellulase composition of the present invention as desired, including stones, pumice, fillers, solvents, enzyme activators, and anti-redeposition agents depending on the eventual use of the composition.

By way of example, stonewashing methods will be described in detail, however, the parameters described are readily modified by the skilled artisan for other applications, i.e., improving the feel and/or appearance of a fabric. The cellulose containing fabric is contacted with the cellulase containing stonewashing composition containing an effective amount of the cellulase by intermingling the treating composition with the stonewashing composition, and thus bringing the cellulase enzyme into proximity with the fabric. Subsequently, the aqueous solution containing the cellulase and the fabric is agitated. If the treating composition is an aqueous solution, the fabric may be directly soaked in the solution. Similarly, where the stonewashing composition is a concentrate, the concentrate is diluted into a water bath with the cellulose containing fabric. When the stonewashing composition is in a solid form, for example a pre-wash gel or solid stick, the stonewashing composition may be contacted by directly applying the composition to the fabric or to the wash liquor.

The cellulose containing fabric is incubated with the stonewashing solution under conditions effective to allow the enzymatic action to confer a stonewashed appearance to the cellulose containing fabric. For example, during stonewashing, the pH, liquor ratio, temperature and reaction time may be adjusted to optimize the conditions under which the stonewashing composition acts. "Effective conditions" necessarily refers to the pH, liquor ratio, and temperature which allow the cellulase enzyme to react efficiently with cellulose containing fabric, in this case to produce the stonewashed effect. However, such conditions are readily ascertainable by one of skill in the art. The reaction conditions effective for the stonewashing compositions of the present invention are substantially similar to well known methods used with corresponding prior art cellulase compositions. Accordingly, it is

within the skill of those in the art to maximize conditions for using the stonewashing compositions according to the present invention.

The liquor ratios during stonewashing, i.e., the ratio of weight of stonewashing composition solution (i.e., the wash liquor) to the weight of fabric, employed herein is generally an amount sufficient to achieve the desired stonewashing effect in the denim fabric and is dependent upon the process used. Preferably, the liquor ratios are from about 4:1 to about 50:1; more preferably from about 5:1 to about 20:1, and most preferably from about 10:1 to about 15:1.

Reaction temperatures during stonewashing with the present stonewashing compositions are governed by two competing factors. Firstly, higher temperatures generally correspond to enhanced reaction kinetics, i.e., faster reactions, which permit reduced reaction times as compared to reaction times required at lower temperatures. Accordingly, reaction temperatures are generally at least about 10°C and greater. Secondly, cellulase is a protein which loses activity beyond a given reaction temperature, which temperature is dependent on the nature of the cellulase used. Thus, if the reaction temperature is permitted to go too high, the cellulolytic activity is lost as a result of the denaturing of the cellulase. While standard temperatures for cellulase usage in the art are generally in the range of 35°C to 65°C, which conditions would also be expected to be suitable for the cellulase of the invention, the optimal temperature conditions should be ascertained according to well known techniques with respect to the specific cellulase used.

Reaction times are dependent on the specific conditions under which the stonewashing occurs. For example, pH, temperature and concentration of cellulase will all effect the optimal reaction time. Generally, reaction times are from about 5 minutes to about 5 hours, and preferably from about 10 minutes to about 3 hours and, more preferably, from about 20 minutes to about 1 hour.

According to yet another preferred embodiment of the present invention, the cellulase of the invention may be employed in a detergent composition. The detergent compositions according to the present invention are useful as pre-wash compositions, pre-soak compositions, or for cleaning during the regular wash or rinse cycle. Preferably, the detergent composition of the present invention comprises an effective amount of cellulase, a surfactant, and optionally includes other ingredients described below.

An effective amount of cellulase employed in the detergent compositions of this invention is an amount sufficient to impart the desirable effects known to be

produced by cellulase on cellulose containing fabrics, for example, depilling, softening, anti-pilling, surface fiber removal, anti-graying and cleaning. Preferably, the cellulase in the detergent composition is employed in a concentration of from about 10 ppm to about 20,000 ppm of detergent.

The concentration of cellulase enzyme employed in the detergent composition is preferably selected so that upon dilution into a wash medium, the concentration of cellulase enzyme is in a range of about 0.01 to about 1000 ppm, preferably from about 0.02 ppm to about 500 ppm, and most preferably from about 0.5 ppm to about 250 ppm total protein. The amount of cellulase enzyme employed in the detergent composition will depend on the extent to which the detergent will be diluted upon addition to water so as to form a wash solution.

The detergent compositions of the present invention may be in any art recognized form, for example, as a liquid, in granules, in emulsions, in gels, or in pastes. Such forms are well known to the skilled artisan. When a solid detergent composition is employed, the cellulase is preferably formulated as granules. Preferably, the granules can be formulated so as to additionally contain a cellulase protecting agent. The granule can be formulated so as to contain materials to reduce the rate of dissolution of the granule into the wash medium. Such materials and granules are disclosed in U.S. Patent No. 5,254,283 which is incorporated herein by reference in its entirety.

The detergent compositions of this invention employ a surface active agent, i.e., surfactant, including anionic, non-ionic and ampholytic surfactants well known for their use in detergent compositions. In addition to the cellulase composition and the surfactant(s), the detergent compositions of this invention can optionally contain one or more of the following components:

<u>Hydrolases Except Cellulase</u>

Suitable hydrolases include carboxylate ester hydrolase, thioester hydrolase, phosphate monoester hydrolase, and phosphate diester hydrolase which act on the ester bond; glycoside hydrolase which acts on glycosyl compounds; an enzyme that hydrolyzes N-glycosyl compounds; thioether hydrolase which acts on the ether bond; and a-amino-acyl-peptide hydrolase, peptidyl-amino acid hydrolase, acyl-amino acid hydrolase, dipeptide hydrolase, and peptidyl-peptide hydrolase which act on the peptide bond. Preferable among them are carboxylate ester hydrolase, glycoside hydrolase, and peptidyl-peptide hydrolase. Suitable hydrolases include

(1) proteases belonging to peptidyl-peptide hydrolase such as pepsin, pepsin B, rennin, trypsin, chymotrypsin A, chymotrypsin B, elastase, enterokinase, cathepsin C, papain, chymopapain, ficin, thrombin, fibrinolysin, renin, subtilisin, aspergillopeptidase A, collagenase, clostridiopeptidase B, kallikrein, gastrisin, cathepsin D., bromelin, keratinase, chymotrypsin C, pepsin C, aspergillopeptidase B, urokinase, carboxypeptidase A and B, and aminopeptidase; (2) glycoside hydrolases (cellulase which is an essential ingredient is excluded from this group) α-amylase, ß-amylase, gluco amylase, invertase, lysozyme, pectinase, chitinase, and dextranase. Preferably among them are α-amylase and ß-amylase. They function in acid to neutral systems, but one which is obtained from bacteria exhibits high activity in an alkaline system; (3) carboxylate ester hydrolase including carboxyl esterase, lipase, pectin esterase, and chlorophyllase. Especially effective among them is lipase.

The hydrolase other than cellulase is incorporated into the detergent composition as much as required according to the purpose. It should preferably be incorporated in an amount of 0.001 to 5 weight percent, and more preferably 0.02 to 3 weight percent, in terms of purified protein. This enzyme should be used in the form of granules made of crude enzyme alone or in combination with other components in the detergent composition. Granules of crude enzyme are used in such an amount that the purified enzyme is 0.001 to 50 weight percent in the granules. The granules are used in an amount of 0.002 to 20 and preferably 0.1 to 10 weight percent. As with cellulases, these granules can be formulated so as to contain an enzyme protecting agent and a dissolution retardant material.

<u>Builders</u>

<u>A. Divalent sequestering agents.</u>

The composition may contain from about 0 to about 50 weight percent of one or more builder components selected from the group consisting of alkali metal salts and alkanolamine salts of the following compounds: phosphates, phosphonates, phosphonocarboxylates, salts of amino acids, aminopolyacetates high molecular electrolytes, non-dissociating polymers, salts of dicarboxylic acids, and aluminosilicate salts. Suitable divalent sequestering gents are disclosed in British Patent Application No. 2 094 826 A, the disclosure of which is incorporated herein by reference.

B. Alkalis or inorganic electrolytes

The composition may contain from about 1 to about 50 weight percent, preferably from about 5 to about 30 weight percent, based on the composition of one or more alkali metal salts of the following compounds as the alkalis or inorganic electrolytes: silicates, carbonates and sulfates as well as organic alkalis such as triethanolamine, diethanolamine, monoethanolamine and triisopropanolamine.

Antiredeposition Agents

The composition may contain from about 0.1 to about 5 weight percent of one or more of the following compounds as antiredeposition agents: polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone and carboxymethylcellulose.

Among them, a combination of carboxymethyl-cellulose and/or polyethylene glycol with the cellulase composition of the present invention provides for an especially useful dirt removing composition.

Bleaching Agents

The use of the cellulase of the present invention in combination with a bleaching agent such as potassium monopersulfate, sodium percarbonate, sodium perborate, sodium sulfate/hydrogen peroxide adduct and sodium chloride/hydrogen peroxide adduct or/and a photo-sensitive bleaching dye such as zinc or aluminum salt of sulfonated phthalocyanine further improves the detergenting effects. Similarly, bleaching agents and bleach catalysts as described in EP 684 304 may be used.

Bluing Agents and Fluorescent Dyes

Various bluing agents and fluorescent dyes may be incorporated in the composition, if necessary. Suitable bluing agents and fluorescent dyes are disclosed in British Patent Application No. 2 094 826 A, the disclosure of which is incorporated herein by reference.

Caking Inhibitors

The following caking inhibitors may be incorporated in the powdery detergent: p-toluenesulfonic acid salts, xylenesulfonic acid salts, acetic acid salts, sulfosuccinic acid salts, talc, finely pulverized silica, amorphous silicas, clay, calcium

silicate (such as Micro-Cell of Johns Manville Co.), calcium carbonate and magnesium oxide.

Masking Agents for Factors Inhibiting the Cellulase Activity

The cellulase composition of this invention are deactivated in some cases in the presence of copper, zinc, chromium, mercury, lead, manganese or silver ions or their compounds. Various metal chelating agents and metal-precipitating agents are effective against these inhibitors. They include, for example, divalent metal ion sequestering agents as listed in the above item with reference to optional additives as well as magnesium silicate and magnesium sulfate.

Cellobiose, glucose and gluconolactone act sometimes as inhibitors. It is preferred to avoid the co-presence of these saccharides with the cellulase as far as possible. In case the co-presence in unavoidable, it is necessary to avoid the direct contact of the saccharides with the cellulase by, for example, coating them.

Long-chain-fatty acid salts and cationic surfactants act as the inhibitors in some cases. However, the co-presence of these substances with the cellulase is allowable if the direct contact of them is prevented by some means such as tableting or coating.

The above-mentioned masking agents and methods may be employed, if necessary, in the present invention.

Cellulase-Activators

The activators may vary depending on the specific cellulase. In the presence of proteins, cobalt and its salts, magnesium and its salts, and calcium and its salts, potassium and its salts, sodium and its salts or monosaccharides such as mannose and xylose, many cellulases are activated and their deterging powers are improved remarkably.

Antioxidants

The antioxidants include, for example, tert-butyl-hydroxytoluene, 4,4'-butylidenebis(6-tert-butyl-3-methylphenol), 2,2'-butylidenebis(6-tert-butyl-4-methylphenol), monostyrenated cresol, distyrenated cresol, monostyrenated phenol, distyrenated phenol and 1,1-bis(4-hydroxy-phenyl)cyclohexane.

Solubilizers

The solubilizers include, for example, lower alcohols such as ethanol, benzenesulfonate salts, lower alkylbenzenesulfonate salts such as p-toluenesulfonate salts, glycols such as propylene glycol, acetylbenzene-sulfonate salts, acetamides, pyridinedicarboxylic acid amides, benzoate salts and urea.

The detergent composition of the present invention can be used in a broad pH range from acidic to alkaline pH. In a preferred embodiment, the detergent composition of the present invention can be used in mildly acidic, neutral or alkaline detergent wash media having a pH of from above 5 to no more than about 12.

Aside from the above ingredients, perfumes, buffers, preservatives, dyes and the like can be used, if desired, with the detergent compositions of this invention. Such components are conventionally employed in amounts heretofore used in the art.

When a detergent base used in the present invention is in the form of a powder, it may be one which is prepared by any known preparation methods including a spray-drying method and a granulation method. The detergent base obtained particularly by the spray-drying method, agglomeration method, dry mixing method or non-tower route methods are preferred. The detergent base obtained by the spray-drying method is not restricted with respect to preparation conditions. The detergent base obtained by the spray-drying method is hollow granules which are obtained by spraying an aqueous slurry of heat-resistant ingredients, such as surface active agents and builders, into a hot space. After the spray-drying, perfumes, enzymes, bleaching agents, inorganic alkaline builders may be added. With a highly dense, granular detergent base obtained such as by the spray-drying-granulation or agglomeration method, various ingredients may also be added after the preparation of the base.

When the detergent base is a liquid, it may be either a homogeneous solution or an inhomogeneous dispersion. For removing the decomposition of carboxymethylcellulose by the cellulase in the detergent, it is desirable that carboxymethylcellulose is granulated or coated before the incorporation in the composition.

The detergent compositions of this invention may be incubated with cellulose containing fabric, for example soiled fabrics, in industrial and household uses at temperatures, reaction times and liquor ratios conventionally employed in these environments. The incubation conditions, i.e., the conditions effective for treating cellulose containing fabrics with detergent compositions according to the present

invention, will be readily ascertainable by those of skill in the art. Accordingly, the appropriate conditions effective for treatment with the present detergents will correspond to those using similar detergent compositions which include known cellulases.

Detergents according to the present invention may additionally be formulated as a pre-wash in the appropriate solution at an intermediate pH where sufficient activity exists to provide desired improvements softening, depilling, pilling prevention, surface fiber removal or cleaning. When the detergent composition is a pre-soak (e.g., pre-wash or pre-treatment) composition, either as a liquid, spray, gel or paste composition, the cellulase enzyme is generally employed from about 0.0001 to about 1 weight percent based on the total weight of the pre-soak or pre-treatment composition. In such compositions, a surfactant may optionally be employed and when employed, is generally present at a concentration of from about 0.005 to about 20 weight percent based on the total weight of the pre-soak. The remainder of the composition comprises conventional components used in the pre-soak, i.e., diluent, buffers, other enzymes (proteases), and the like at their conventional concentrations.

It is contemplated that compositions comprising cellulase enzymes described herein can be used in home use as a stand alone composition suitable for restoring color to faded fabrics (see, for example, U.S. Patent No. 4,738,682, which is incorporated herein by reference in its entirety) as well as used in a spot-remover and for depilling and antipilling (pilling prevention).

The use of the cellulase according to the invention may be particularly effective in feed additives and in the processing of pulp and paper. These additional industrial applications are described in, for example, PCT Publication No. 95/16360 and Finnish Granted Patent No. 87372, respectively.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given with the understanding that they are being offered to illustrate the present invention and should not be construed in any way as limiting its scope.

EXAMPLES

Example 1
Preparation of Genomic DNA Encoding EGIII-Like Cellulases

Genomic DNA was prepared for several different microorganisms for the purpose of undertaking a PCR reaction to determine whether EGIII-like cellulases are encoded by the DNA for a particular organism.

Genomic DNA is obtained from *Acremonium brachypenium* deposit no. CBS 866.73; *Chaetomium brasillience* deposit no. CBS 140.50; *Chaetomium vitellium* deposit no. CBS 250.85; *Emericella desertoru* deposit no. CBS 653.73; *Fusarium equiseti* deposit no. CBS 185.34; *Gliocladium roseum* deposit no. CBS 443.65; *Humicola grisea var. thermoidia* deposit no. CBS 225.63; *Myceliopthora thermophila* deposit no. ATCC 48102-48104; *Penicillium notatum* deposit no. ATCC 9178, 9179; *and Phanerochaete chrysosporium* deposit no. ATCC 28326 and isolated according to standard methods.

PCR was performed on a standard PCR machine such as the PCT-150 MicroCycler from MJ Research Inc. under the following conditions:

1) 1 minute at 98°C for 1 cycle;
2) 1 minute at 94°C,
   90 seconds at 40°C,
   1minute at 72°C
3) repeat step 2 for 30 cycles
4) 7 minutes at 72°C for 1 cycle
5) lower temperature to 15°C for storage and further analysis.

The following DNA primers were constructed for use in amplification of EGIII-like genes from the libraries constructed from the various microorganisms. All symbols used herein for protein and DNA sequences correspond to IUPAC IUB Biochemical Nomenclature Commission codes.

BOX1: primers coding for (N/Q)NLWG

forward primer	FRG001: AAY AAY YTN TGG GG
forward primer	FRG002: CAR AAY YTN TGG GG

BOX1': primers coding for NNN(F/L/Y/I/L/N/K)WG

forward primer	FRG010: AAY AAY AAY HWI TGG GG

BOX2: primers coding for ELMIW

forward primer	FRG003: GAR YTN ATG ATH TGG
reversed primer	FRG004: CCA DAT CAT NAR YTC

BOX2': primers coding for YELMIW

forward primer	FRG011: TAY GAR YTI ATG ATH TGG
reversed primer	FRG012: CCA DAT CAT IAR YTC RTA

BOX3: primers coding for GTE(P/C)FT

reversed primer	FRG005: GTR AAN GGY TCR GTR CC
reversed primer	FRG006: GTR AAN GGY TCR GTY CC
reversed primer	FRG007: GTR AAN GGY TCY GTR CC
reversed primer	FRG008: GTR AAN GGY TCY GTY CC
reversed primer	FRG009: GTR AAR CAY TCN GTN CC

PCR conditions for PWO polymerase (Boehringer Mannheim, Cat # 1644-947) comprise a 100 microliter solution made of 10 microliter of 10X reaction buffer (10X reaction buffer comprising 100mM Tris HCl, pH 8-8.5; 250 mM KCl; 50 mM $(NH_4)_2SO_4$; 20 mM $MgSO_4$); 0.2 mM each of dATP, dTTP, dGTP, dCTP (final concentration), 1 microliter of 100 nanogram/microliter genomic DNA, 1 microliter of PWO at 1 unit per microliter, 500 mM primers (final concentration) and water to 100 microliters. The solution is overlaid with mineral oil.

The PCR strategy was as follows: forward primers for BOX1 and BOX1' were combined with reversed primers from BOX3 in a mixture with the desired genomic DNA sample and run on a gel to obtain fragments in the 400-1000 base pair range. The obtained fragments were then pooled and the pool split into two approximately equal portions. The first pool was combined with the forward primers from BOX1 and BOX1' along with the reversed primer from BOX2. The second pool was combined with the forward primer from BOX2 along with the reversed primers from BOX3. Fragments having the approximate size relative to an EGIII-like cellulase considering the location of the primers within the gene, in this case corresponding to those between 250-500 base pairs, were isolated and sequenced.

From the sequenced fragments, it was possible to use the RAGE technique (rapid amplification of genomic ends) to rapidly obtain the sequence of the full length gene. Full length genes were obtained and are provided with several additional EGIII-like cellulase sequences in Fig. 3. As shown in Fig. 3, full length

genes isolated from *Hypocrea schweinitzii, Aspergillus aculeatus, Aspergillus kawachii (1), Aspergillus kawachii (2), Aspergillus oryzae, Humicola grisea, Humicola insolens, Chaetomium brasilliense, Fusarium equiseti, Fusarium javanicum (1), Fusarium javanicum (2), Gliocladium roseum (1), Gliocladium roseum (2), Gliocladium roseum (3), Gliogladium roseum (4), Memnoniella echinata, Actinomycete 11AG8, Streptomyces lividans CelB, Rhodothermus marinus, Emericella desertoru,* and *Erwinia carotovara* all comprise significant homology EGIII from *Trichoderma reesei.*

The isolated and partially sequenced DNA and the corresponding amino acid sequences (of approximately 100 residues) were analyzed to determine their relationship to EGIII. The results of this sequence alignment are shown in Fig. 3. As shown in Fig. 3, significant sequence homology exists between the peptides encoded by the obtained DNA fragments and corresponding peptide sequences from EGIII. The high homology and strong conservation of residues corresponding to peptides (a), (b), (c) and/or (d), as in EGIII, identify the genes as coding an EGIII-like cellulase from each of the organisms.

Example 2

Temperature Stability Testing of EGIII and an EGIII Homolog from *Hypocrea schweinitzii*

EGIII and an EGIII-like cellulase derived from *Hypocrea schweinitzii* were tested to determine their stability under temperature stress. 0.3 mg/ml of enzyme was tested in 0.1M MOPS, at pH 7.3, 48°C and the activity on oNPC measured and compared over time. The experiment was run two times. The natural log of the activity was plotted against time of incubation, and the rate constant for inactivation obtained from the slope of the straight line. Results for various mutants are provided in Table 1.

Table 1

Half Life of EGIII and a Homolog

Trichoderma reesei EGIII	*EGIII Homolog from Hypocrea schweinitzii*
20.2	3.40
21.2	3.90

As shown in Table 1, the half life of EGIII from *T. reesei* is significantly greater than that of the EGIII homolog from *Hypocrea schweinitzii*.

Example 3

Wash Tests With EGIII and an EGIII-Like Cellulase From *Hypocrea schweinitzii*

EGIII was compared to a homologous enzyme derived from from *Hypocrea schweinitzii*. The amino acid sequence of the enzyme from *Hypocrea schweinitzii* is provided in Fig. 3 in alignment with the sequence of EGIII. As shown in Fig. 3, the amino acid sequence of the two enzymes is identical except for the residues in bold corresponding to positions 11, 12, 23, 27, 32, 55, 57, 79, 81, 93, 107, 159, 179, 183 and 204. The test was run as follows:

Three different enzyme mixtures (a) EGIII, (b) an EGIII homolog derived from *Hypocrea schweinitzii,* and (c) a combination of the two enzymes were prepared and mixed separately with a standard LAS containing granular detergent (4g/l) in water having a hardness of 70 ppm $CaCO_3$ (2:1 Ca:Mg) at 40°C in a Terg-o-Tometer with cotton swatches. The agitation was 125 rpm and the test was run for 2.5 hours. After the test, the swatches were removed from the Terg-o-Tometer, dried in a tumble drier and the level of the depilling compared to a panel of fabrics pilled to varying extents. Fig. 4 shows the depilling performance of the enzymes against the concentration of enzyme. As shown in Fig. 4., the EGIII-like enzyme from *Hypocrea schweinitzii* showed no depilling performance at any concentration. By contrast, EGIII showed depilling performance which increased in accordance with the enzyme concentration. The equivalent performance of EGIII spiked into the *Hypocrea schweinitzii* broth containing the EGIII-like enzyme shows that it is not a component of the broth which prevents performance of the EGIII-like enzyme but, instead, the enzyme itself which has poor stability and performance.

The results of this experiment illustrate that the stability of the EGIII-like enzyme from *Hypocrea schweinitzii* is far inferior to EGIII. In fact, the related enzyme has no activity in the LAS containing detergent whereas EGIII retains excellent activity. These results thus show that the 14 residues which differ between the two enzymes are responsible for surfactant stability and thus are

critical to improving the stability of EGIII. Accordingly, appropriate modification of some or all of these residues in EGIII is very likely to result in improved enzyme performance in the presence of surfactant.

WE CLAIM:

1. A variant EGIII-like cellulase comprising a substitution at a surfactant sensitive residue.

2. The cellulase of claim 1, wherein said variant comprises a substitution or deletion at a position corresponding to one or more of residues 11, 12, 23, 27, 32, 51, 55, 57, 79, 81, 93, 107, 159, 179, 183 and/or 204 in EGIII from *Trichoderma reesei*.

3. The cellulase according to claim 2, wherein said substitution comprises replacing said residue to correspond to one or more of residues L11, I12, W23, T27, T32, A51, S55, G57, S79, A81, S93, N107, S159, T179, N183 and/or A204 or a conservative substitution thereof.

4. The cellulase according to claim 1, said cellulase being derived from a fungus, bacteria or Actinomycete.

5. The cellulase according to claim 1, wherein said cellulase is an endoglucanase.

6. The cellulase according to claim 1, wherein said fungus is a filamentous fungus.

7. The cellulase according to claim 6, wherein said filamentous fungus belongs to Euascomycete.

8. The cellulase according to claim 11, wherein said Euascomycete is *Aspergillus spp., Gliocladium spp., Fusarium spp., Acremonium spp., Myceliophtora spp., Verticillium spp., Myrothecium spp.,* or *Penicillium spp.*

9. A DNA encoding the cellulase according to claim 1.

10. A vector comprising the DNA of claim 9.

11. A host cell transformed with the vector of claim 10.

12. A method of producing a cellulase comprising the steps of:

(a) culturing the host cell according to claim 11 in a suitable culture medium under suitable conditions to produce cellulase;

(b) obtaining said produced cellulase; and optionally

(c) purifying said cellulase to provide a purified cellulase product.

13. A detergent composition comprising a surfactant and a cellulase, wherein said cellulase comprises a variant EGIII-like cellulase comprising a substitution at a surfactant sensitive residue.

14. The detergent of claim 13, wherein said variant EGIII cellulase comprises a substitution or deletion at a position corresponding to one or more of

residues 11, 12, 23, 27, 32, 51, 55, 57, 79, 81, 93, 107, 159, 179, 183 and/or 204 in EGIII from *Trichoderma reesei*.

15. The detergent according to claim 14, wherein said substitution comprises replacing said residue so as to correspond to one or more of L11, I12, W23, T27, T32, A51, S55, G57, S79, A81, S93, N107, S159, T179, N183 and/or A204 or a conservative substitution thereof.

16. The detergent according to claim 13, wherein said detergent is a laundry detergent.

17. The detergent according to claim 13, wherein said detergent is a dish detergent.

18. The use of the variant EGIII-like cellulase according to claim 1 in the treatment of a cellulose containing textile.

19. The use of the EGIII-like cellulase according to claim 1 as a feed additive.

20. The use of the EGIII-like cellulase according to claim 1 in the treatment of wood pulp.

21. The use of the EGIII-like cellulase according to claim 1 in the reduction of biomass to glucose.

22. The use of the EGIII-like cellulase according to claim 1 in the stonewashing or indigo dyed denim.

Amino Acid Sequence of EGIII

MKFLQVLPALIPAALAQTSCDQWATFTGNGYTVSNNLWGASAGSGFGCVTAVSLSGGAHADWQWS
GGQNNVKSYQNSQIAIPQKRTVNSISSMPTTASWSYSGSNIRANVAYDLFTAANPNHVTYSGDYE
LMIWLGKYGDIGPIGSSQGTVNVGGQSWTLYYGYNGAMQVYSFVAQTNTTNYSGDVKNFFNYLRD
NKGYNAAGQYVLSYQFGTEPFTGSGTLNVASWTASIN

FIG._1

DNA Sequence of EGIII Without Introns

ATGAAGTTCCTTCAAGTCCTCCCTGCCCTCATACCGGCCGCCCTGGCCCAAACCAGCTGTGACCA
GTGGGCAACCTTCACTGGCAACGGCTACACAGTCAGCAACAACCTTTGGGGAGCATCAGCCGGCT
CTGGATTTGGCTGCGTGACGGCGGTATCGCTCAGCGGCGGGGCCTCCTGGCACGCAGACTGGCAG
TGGTCCGGCGGCCAGAACAACGTCAAGTCGTACCAGAACTCTCAGATTGCCATTCCCCAGAAGAG
GACCGTCAACAGCATCAGCAGCATGCCCACCACTGCCAGCTGGAGCTACAGCGGGAGCAACATCC
GCGCTAATGTTGCGTATGACTTGTTCACCGCAGCCAACCCGAATCATGTCACGTACTCGGGAGAC
TACGAACTCATGATCTGGCTTGGCAAATACGGCGATATTGGGCCGATTGGGTCCTCACAGGGAAC
AGTCAACGTCGGTGGCCAGAGCTGGACGCTCTACTATGGCTACAACGGAGCCATGCAAGTCTATT
CCTTTGTGGCCCAGACCAACACTACCAACTACAGCGGAGATGTCAAGAACTTCTTCAATTATCTC
CGAGACAATAAAGGATACAACGCTGCAGGCCAATATGTTCTTAGCTACCAATTTGGTACCGAGCC
CTTCACGGGCAGTGGAACTCTGAACGTCGCATCCTGGACCGCATCTATCAAC

FIG._2

```
                            1                                                         60
T._reesei                   M........KF.LQVLPALIPAALAQTS................CDQWATFTGNG..YTV
H._schweinitzii             M........KF.LQVLPAILPAALAQTS................CDQYATFSGNG..YIV
A._aculeatus__*             M........KAFHL.LAALAGAAVAQQAQ..............LCDQYATYTGGV..YTI
A._kawachii__*              M........KLSMT.LSLFAATAMGQT................MCSQYDSASSPP..YSV
A._kawachii_2               M........KAFHL.LAALSGAAVAQQAQ..............LCDQYATYTGGV..YTI
A._oryzae__*                M........KLSLA.LATLVATAFSQE................LCAQYDSASSPP..YSV
H._grisei                   M.......LKSALLLGAAAVSVQSASIPTIPANLEPRQIR.SLCELYGYWSGNG..YEL
H._insolens__*              M.......LKSALLLGPAAVSVQSASIPTIPANLEPRQIR.SLCELYGYWSGNG..YEL
Chaetomium_brasiliense      M........KLTLVLFVSSLA......AATPLGWRERQQQVSLCGQSSSWSGNG..YQL
F._equseti                  M........KSTLLLAGAFAPLAFAKD................LCEQYGYLSSDG..YSL
F._javanicum_1              M........KSAIVA.ALAGLAAASPTRLIPRGQ........FCGQWDSETAGA..YTI
F._javanicum_2              M........K..FFGVVSASLAATAVATPTTPTETIEKRDTTWCDAFGSLATSG..YTV
G._roseum_Rj_1              M........KANIVILSLFAPLAAVAQT...............LCGQYSSNTQGG..YIF
G._roseum_Rj_2              M........KSIISFFGLATLVAAAPSQNPTRTQPLEKRATTLCGQWDSVETGG..YTI
G._roseum_PA_3              M........KFQLLSLTAFAPLSLAA.................LCGQYQSQSQGG..YIF
G._roseum_Rj_4              M........KTGIAYLAAVLPLA.MAES...............LCDQYAYLSRDG..YNF
Memnoniella_echinata        M........KVAAL.LVALSPLAF.AQS...............LCDQYSYYSSNG..YEF
Emericella_desertoru        M........K..LLALSLVSLASAASAASIL.SNTFTRRSD.FCGQWDTATVGN..FIV
Actinomycete_11AG8          MRS......HPRS..ATM.TVLVVLASLGALLTAAAPAQANQQICDRYGTTTIQD.RYVV
S._lividans_CelB__*         MRTLRPQARAPRGLLAALGAVLAAFALVSSLVTAAAPAQADTTICEPFGTTTIQG.RYVV
Rhodothermus_marinus__*     MNVMR..AVLVLSLLLLFGCDWL.FPDGDNGKEPEPEPEPTVELCGRWDARDVAGGRYRV
Erwinia_carot___*           MQTVNTQPHRIFRVLLPAVFSSLLLSSLTVSAASSSNDADKLYF.........GNNKYYL
```

FIG._3A

```
                            61                                                        120
               T._reesei    SNNLWGASAGSGF..GCV.TAVSLSGG.ASWHADWQWSGGQNNVKSYQNS..........
          H._schweinitzii   SNNLWGASAGSGF..GCV.TSVSLNGA.ASWHADWQWSGGQNNVKSYQNV..........
          A._aculeatus__*   NNNLWGKDAGSG..SQCTTVNSASSAG.TSWSTKWNWSGGENSVKSYANS..........
           A._kawachii__*   NQNLWGEYQGTG..SQCVYVDKLSSSG.ASWHTKWTWSGGEGTVKSYSNS..........
            A._kawachii_2   NNNLWGKDAGSG..SQCTTVNSASSAG.TSWSTKWNWSGGENSVKSYANS..........
             A._oryzae__*   NNNLWGQDSGTGFTSQCVYVDNLSSSG.AAWHTTWTWNGGEGSVKSYSNS..........
               H._grisei    LNNLWGKDTATS.GWQCTYLDGTNNGG.IQWNTAWEWQGAPDNVKNYPYV..........
           H._insolens__*   LNNLWGKDTATS.GWQCTYLDGTNNGG.IQWSTAWEWQGAPDNVKSYPYV..........
  Chaetomium_brasiliense    NNNLWGQSRATS.GSQCTYLDSSSNSG.IHWHTTWTWEGGEGEVKSYAYS..........
               F._equseti   NNNVWGKDSGTGD..QCTHVNWNNANG.AGWDVEWNWSGGKDNVKSYPNS..........
          F._javanicum_1    YNNLWGKDNAES.GEQCTTNSGEQSDGSIAWSVEWSWTGGQGQVKSYPNA..........
          F._javanicum_2    YHNNWGKGDATS.GSQCTTFTSVSNNNFV.WSTSWTWAGGAGKVKSYSNV..........
          G._roseum_Rj_1    NNNMWGMGSGSGS..QCTYVDKVWAEG.VAWHTDWSWSGGDNNVKSYPYS..........
          G._roseum_Rj_2    YNNLWGQDNG.S.GSQCLTVEGV.TDGLAAWSSTWSWSGGSSSVKSYSNA..........
          G._roseum_PA_3    NNNKWGQGSGSGS..QCLTIDKTWDSN.VAFHADWSWSGGTNNVKSYPNA..........
          G._roseum_Rj_4    NNNEWGAATGTGD..QCTYVDSTSSGG.VSWHSDWTWSGSESEIKSYPYS..........
   Memnoniella_echinata     NNNMWGRNSGQGN..QCTYVDYSSPNG.VGWRVNWNWSGGDNNVKSYPYS..........
   Emericella_desertoru     YNNLWGQDNADS.GSQ..TGVDSANGNSISWHTTWSWSGGSSSVKSYANA..........
     Actinomycete_11AG8     QNNRWGTSAT.....QCINVT..GNGFEITQADGS..VPTNGAPKSYPSVYDGCHYG...
     S._lividans_CelB__*    QNNRWGSTAP.....QCVTAT..DTGFRVTQADGS..APTNGAPKSYPSVFNGCHYT...
  Rhodothermus_marinus__*   INNVWGAETA.....QCIEVGLETGNFTITRADHD..NGNNVA..AYPAIYFGCHWAPAR
       Erwinia_carot___*    FNNVWGKDEIKGWQQTIFYNSPISMG....WN..WHWPSSTHSVKAYPSLVSGWHWTAG.
```

FIG._3B

```
                        121                                                        180
T._reesei               .QIAIP.QKRTVNSISSMPTTASW...SYSGSNIRANVAYDL.FTAANPNHVTYSGDYEL
H._schweinitzii         .QINIP.QKRTVNSIGSMPTTASW...SYSGSDIRANVAYDL.FTAANPNHVTYSGDYEL
A._aculeatus__*         .GLTF..NKKLVSQISQIPTTARW.S..YDNTGIRADVAYDL.FTAADINHVTWSGDYEL
A._kawachii__*          .GLTF..DKKLVSDVSSIPTSVTW.SQD..DTNVQADVSYDL.FTAANADHATSSGDYEL
A._kawachii_2           .GLSF..NKKLVSQISHIPTAARW.S..YDNTCIRRGRAYDL.FTAADINHVTWSGDYEL
A._oryzae__*            .AVTF..DKKLVSDVQSIPTDVEW.SQDFTNTNVNADVAYDL.FTAADQNHVTYSGDYEL
H._grisei               .GKQIQRGRK.ISDINSMRTSVSW...TYDRTDLRANVAYDV.FTARDPDHPNWGGDYEL
H._insolens__*          .GKQIQRGRK.ISDINSMRTSVSW...TYDRTDIRANVAYDV.FTARDPDHPNWGGDYEL
Chaetomium_brasiliense  .GRQVSTGLT.IASIDSMQTSVSW...EYNTTDIQANVAYDI.FTAEDPDHEHSSGDYEL
F._equseti              .ALLIGEDKKTISSITNMQSTAEW...KYSGDNLRADVAYDL.FTAADPNHETSSGEYEL
F._javanicum_1          .VVEI..EKKTLGEVSSIPSA..W.DWTYTGNGIIANVAYDL.FTSSTESGDA...EYEF
F._javanicum_2          .ALEK..INKKISDIKSVSTR..W.IWRYTGTKMIANVSYDL.WFAPTASSNN...AYEI
G._roseum_Rj_1          .GRELGT.KRIVSSIKSISSGADW...DYTGSNLRANAAYDI.FTSANPNHATSSGDYEV
G._roseum_Rj_2          .VLSA..EAARISAISSIPSK..W.EWSYTGTDIVANVAYDL.FSNTDCGDTP...EYEI
G._roseum_PA_3          .GLEFSR.GKKVSSIGTINGGADW...DYSGSNIRANVAYGI.FTSADPNHVTSSGDYEL
G._roseum_Rj_4          .GLDLPE.KKIVTSIGSISTGAEW...SYSGSDIRADVAYDT.FTAADPNHATSSGDYEV
Memnoniella_echinata    .GRQLPT.KRIVSWIGSLPTTVSW...NYQGNNLRANVAYDL.FTAANPNHPNSSGDYEL
Emericella_desertoru    .AYQF..TSTKLNSLSSIPTS..W.KWQYSTTDIVANVAYDL.FTSSSAGGDS...EYEI
Actinomycete_11AG8      ...NCAPRTTLPMRISSIGSAPSSVSYRYTGNGVY.NAAYDIWLDPTPRTNGVNR..TEI
S._lividans_CelB__*     ...NCSPGTDLPVRLDTVSAAPSSISYGFVDGAVY.NASYDIWLDPTARTDGVNQ..TEI
Rhodothermus_marinus__* AIRDCAARAGAVRRAHELDVTP.......ITTGRW.NAAYDIWFSPVTNSGNGYSGGAEL
Erwinia_carot___*       ....YTENSGLPIQLSSNKSITSNVTYSIKATGTY.NAAYDIWFHTTDKANWDSSPTDEL
```

FIG._3C

```
                          181                                                      240
             T._reesei    MIWLGKYGDIGPIGSS....QGTVNVGGQSWTLYYGYNGAMQV......YSFVAQT.NTT
       H._schweinitzii    MIWLGKYGDIGPIGSS....QGTVNVGGQTWTLYYGYNGAMQV......YSFVAQS.NTT
        A._aculeatus__*   MIWLARYGGVQPIGSQ....IATATVDGQTWELWYG......ANGSQKTYSFVAPT.PIT
         A._kawachii__*   MIWLARYGSVQPIGKQ....IATATVGGKSWEVW..YGTSTQAGAEQKTYSFVAGS.PIN
          A._kawachii_2   MIWLARYGGVQPLGSQ....IATATVEGQTWELWYG......VNGAQKTYSFVAAN.PIT
           A._oryzae__*   MIWLARYGTIQPIGTQ....IDTATVEGHTWELWFTYGTTIQAGAEQKTYSFVSAT.PIN
              H._grisei   MIWLARYGGIYPIGTF....HSQVNLAGRTWDLWTGYNGNMRV......YSFLPPSGDIR
         H._insolens__*   MIWLARYGGIYPIGTF....HSQVNLAGRTWDLWTGYNGNMRV......YSFLPPSGDIR
  Chaetomium_brasiliense  MIWLARYNNVSPIGSS....VATATVGGDTWDLFAGANGDMEV......YSFVAENT.MN
             F._equseti   MVWLARIGGVQPIGSL....QTSVTIEGHTWELWVGMNGSMKV......FSFVAPT.PVN
          F._javanicum_1  MIWLSALGGAGPISNDGSP.VATAELAGTSWKLYQGKNNQMTV......FSFVAESDV.N
          F._javanicum_2  MIWVGAYGGALPISTPGKGVIDRPTLAGIPWDVYKGPNGDVTV......ISFVASSNQ.G
          G._roseum_Rj_1  MIWLANLGGLTPIGSP....IGTVKAAGRDWELWDGYNGAMRV......YSFVAPS.QLN
          G._roseum_Rj_2  MIWLSALGGAGPISSTGSS.IATVTIAGASWNLWQGQNNQMAV......FSFVAESDQ.K
          G._roseum_PA_3  MIWLGKLGDIYPIGNS....IGRVEAANREWDFLVGYNGAMKV......FSFVAPS.PVT
          G._roseum_Rj_4  MIWLANLGGLTPIGSP....IGTVKAAGRDWELWDGYNGAMRV......YSFVAPS.QLN
    Memnoniella_echinata  MIWLGRLGNVYPIGNQ....VATVNIAGQQWNLYYGYNGAMQV......YSFVSPN.QLN
    Emericella_desertoru  MIWLAALGGAGPISSTGSS.IATVTLGGVTWSLYSGPNGSMQV......YSFVASSTT.E
      Actinomycete_11AG8  MIWFNRVGPVQPIGSP....VGTAHVGGRSWEVWTGSNGSNDVI......SFLAPSA.IS
       S._lividans_CelB__*  MIWFNRVGPIQPIGSP....VGTASVGGRTWEVWSGGNGSNDVL......SFVAPSA.IS
  Rhodothermus_marinus__*  MIWLNWNGGVMPGGSR....VATVELAGATWEVWYADWDWNYIA......YRRTTPT.TS
      Erwinia_carot___*   MIWLNDTNA.....GPAGDYIETVFLGDSSWNVFKGWINADN.GGGWNVFSFVHTSGTNS
```

FIG._3D

```
                           241                                                         300
               T._reesei   NYSGDVKNFFNYLRDNKGYNAAGQYV..LSYQFGTEPF..TGSGT.LNVASWTASI.N..
        H._schweinitzii   SYSGDVKNFFNYLRDNKGYNAGGQYV..LSYQFGTEPF..TGSGT.LNVASWTASI.N..
          A._aculeatus__*  SFQGDVNDFFKYLTQNHGFPASSQYLI..TLQFGTEPF..TGGPATLSVSNWSASVQQAG
           A._kawachii__*  SWSGDIKDFFNYLTQNQGFPASSQHLI..TLQCGTEPF..TGGPATFTVDNWTASVN...
           A._kawachii_2   SFQGDINDFFKYLTQNHGFPASSQYLIILALQFGTEPF..TGGPATLNVADWSASVQ...
             A._oryzae__*  TFGGDIKKFFDYITSKHSFPASAQYLI..NMQFGTEPFFTTGGPVTFTVPNWTASVN...
               H._grisei   DFSCDIKDFFNYLERNHGYPAREQNLIV..YQVGTECF..TGGPARFTCRDFRADL....
           H._insolens__*  DFSCDIKDFFNYLERNHGYPAREQNLIV..YQVGTECF..TGGPARFTCRDFRADL....
 Chaetomium_brasiliense   SFSGDVKDFFDYLEQNVGFPVDDQYLLV..FELGSEAF..TGGPATLSVSQFSANI....
              F._equseti   NFNADIKQFWDYLTKSQNFPADNQYL..LTFQFGTEPF..TGDNAKFTVTNFNAHLK...
          F._javanicum_1   NFCGDLADFTDYLVDNHGVSSSQ...ILQSVGAGTEPF..EGTNAVFTTNNYHADVE...
          F._javanicum_2   NFQADLKEFLNYLTSKQGLPSNY...VATSFQAGTEPF..EGTNAVLKTSAYTISVN...
          G._roseum_Rj_1   SFDGEIMDFFYVVKDMRGFPADSQHL..LTVQFGTEPI..SGSGAKFSVSHWSAKLG...
          G._roseum_Rj_2   SFSGDLNDFIQYLVDSQGYSGSQ...CLYSIGAGTEPF..TGTDAEFITTGYSVSVSAGD
          G._roseum_PA_3   LFDGNIMDFFYVMRDMQGYPMDKQYL..LSLQFGTEPF..TGSNANFSCWYFGAKIK...
          G._roseum_Rj_4   SFDGEIMDFFYVVKDMRGFPADSQHL..LTVQFGTEPI..SGSGAKFSVSHWSAKLG...
 Memnoniella_echinata    YFSGNVKDFFTYLQYNRAYPADSQYL..ITYQFGTEPF..TGQNAVFTVSNWSAQQNN..
 Emericella_desertoru    SFSADLMDFINYLAENQGLSSSQ...YLTHVQAGTEPF..TGTDATLTVSSYSVSVS...
    Actinomycete_11AG8   SWSFDVKDFVD.QAVSHGLATPDWYLT..SIQAGFEPW...EGGTGLAVNSFSSAVNAG.
       S._lividans_CelB__* GWSFDVMDFVR.ATVARGLAENDWYLT..SVQAGFEPW...QNGAGLAVNSFSSTVETGT
 Rhodothermus_marinus__* VSELDLKAFID.DAVARGYIRPEWYLH..AVETGFELW...EGGAGLRTADFSVTVQ...
       Erwinia_carot___*   A.SLNIRHFTDYLVQTKQWMSDEKYIS..SVEFGTEIF...GGDGQIDITEWRVDVK...
```

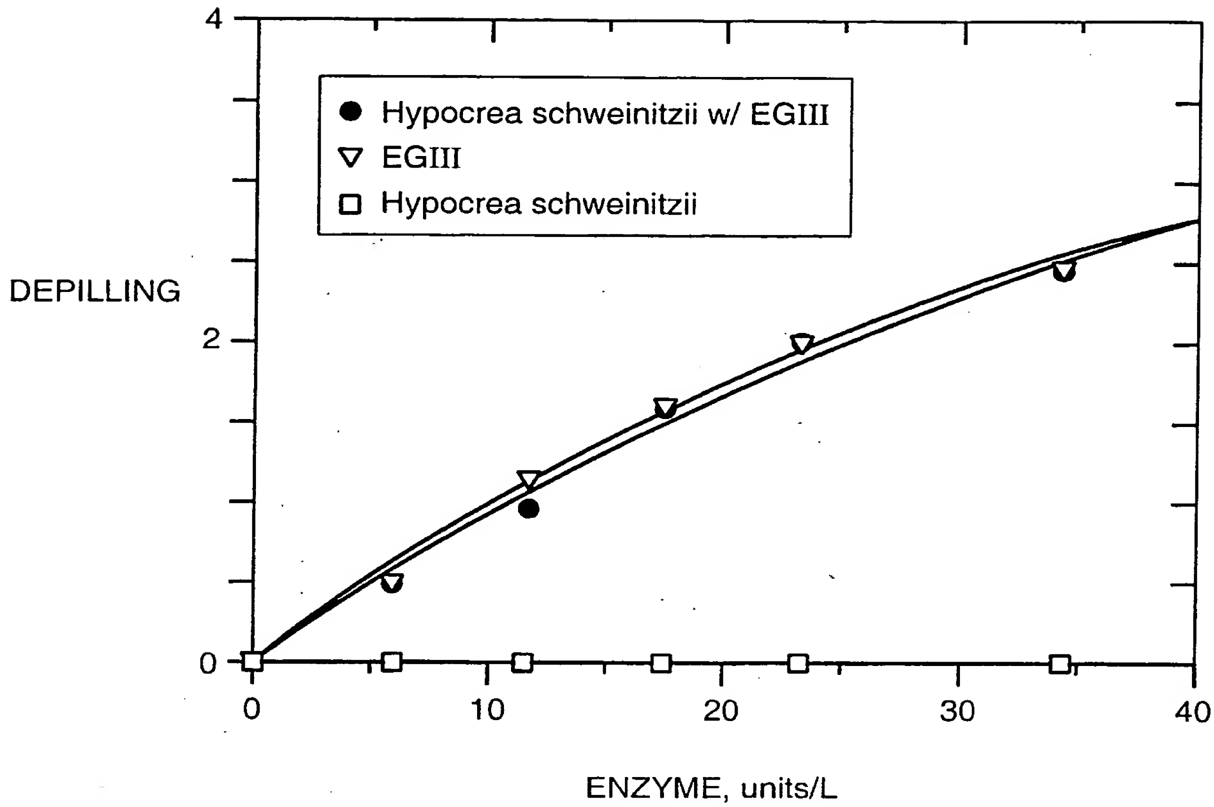
FIG._3E

```
                          301                                                        360
T._reesei                 ............................................................
H._schweinitzii           ............................................................
A._aculeatus__*           F.........................................EPWQNGAGLAVNSF....
A._kawachii__*            ............................................................
A._kawachii_2             ............................................................
A._oryzae__*              ............................................................
H._grisei                 ............................................................
H._insolens__*            ............................................W...............
Chaetomium_brasiliense    ............................................W...............
F._equseti                .............................................A..............
F._javanicum_1            ............................................................
F._javanicum_2            ............................................................
G._roseum_Rj_1            ............................................................
G._roseum_Rj_2            SGCDETTTSSQAQSSTVETSTATQPQS...SSTVVPTVTLS.QPSNESTTTPVQSQ....
G._roseum_PA_3            ............................................................
G._roseum_Rj_4            ............................................................
Memnoniella_echinata      ............................................................
Emericella_desertoru      ............................................................
Actinomycete_11AG8        ..GGNGGTPGTPAACQVSYSTHTWPGGFTVDTTITNTGSTPVDGWELDFTLPAGHTVTSA
S._lividans_CelB__*       PGGTDPGDPGGPSACAVSYGTNVWQDGFTADVTVTNTGTAPVDGWQLAFTLPSGQRITNA
Rhodothermus_marinus__*   ............................................................
Erwinia_carot___*         ............................................................
```

FIG._3F

```
                           361                                                       419
T._reesei                  ...........................................................
H._schweinitzii            ...........................................................
A._aculeatus__*            ......SSTV.................................................
A._kawachii__*             ...........................................................
A._kawachii_2              ...........................................................
A._oryzae__*               ...........................................................
H._grisei                  ...........................................................
H._insolens__*             ...........................................................
Chaetomium_brasiliense     ...........................................................
F._equseti                 ..........................................................Y
F._javanicum_1             ...........................................................
F._javanicum_2             ...........................................................
G._roseum_Rj_1             ...........................................................
G._roseum_Rj_2             ......PSSVETTPTAQPQSSSVQTTTTAQA....QPTSGTGCSRRRKRR......AVV
G._roseum_PA_3             ...........................................................
G._roseum_Rj_4             ...........................................................
Memnoniella_echinata       ...........................................................
Emericella_desertoru       ...........................................................
Actinomycete_11AG8         WNALISPASGAVTARSTGSNGRIAANGGTQSFGFQGTSSGTGFNAPAGGRLNGTSCTVR
S._lividans_CelB__*        WNASLTPSSGSVTATGASHNARIAP.GGSLSFGFQGTYGGA.FAEPTGFRLNGTACTTV
Rhodothermus_marinus__*    ...........................................................
Erwinia_carot___*          ...........................................................
```

FIG._3G



Hypocrea schweinitzii w/ EGIII
EGIII
Hypocrea schweinitzii
DEPILLING
4
2
0
0
10
20
30
40
ENZYME, units/L

FIG._4

SEQUENCE LISTING

<110> Fowler, Timothy

<120> Improved EGIII-Like Celluase
Compositions, DNA Encoding Such EGIII Compositions and
Methods for Obtaining Same

<130> GC547-PCT

<140> 09/146,729
<141> 1998-09-03

<160> 23

<170> FastSEQ for Windows Version 3.0

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<212> PRT
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Gln Thr Ser Cys Asp Gln Trp Ala Thr Phe Thr Gly Asn Gly Tyr Thr
            20                  25                  30
Val Ser Asn Asn Leu Trp Gly Ala Ser Ala Gly Ser Gly Phe Gly Cys
        35                  40                  45
Val Thr Ala Val Ser Leu Ser Gly Gly Ala His Ala Asp Trp Gln Trp
    50                  55                  60
Ser Gly Gly Gln Asn Asn Val Lys Ser Tyr Gln Asn Ser Gln Ile Ala
65                  70                  75                  80
Ile Pro Gln Lys Arg Thr Val Asn Ser Ile Ser Ser Met Pro Thr Thr
                85                  90                  95
Ala Ser Trp Ser Tyr Ser Gly Ser Asn Ile Arg Ala Asn Val Ala Tyr
            100                 105                 110
Asp Leu Phe Thr Ala Ala Asn Pro Asn His Val Thr Tyr Ser Gly Asp
        115                 120                 125
Tyr Glu Leu Met Ile Trp Leu Gly Lys Tyr Gly Asp Ile Gly Pro Ile
    130                 135                 140
Gly Ser Ser Gln Gly Thr Val Asn Val Gly Gly Gln Ser Trp Thr Leu
145                 150                 155                 160
Tyr Tyr Gly Tyr Asn Gly Ala Met Gln Val Tyr Ser Phe Val Ala Gln
                165                 170                 175
Thr Asn Thr Thr Asn Tyr Ser Gly Asp Val Lys Asn Phe Phe Asn Tyr
            180                 185                 190
Leu Arg Asp Asn Lys Gly Tyr Asn Ala Ala Gly Gln Tyr Val Leu Ser
        195                 200                 205
Tyr Gln Phe Gly Thr Glu Pro Phe Thr Gly Ser Gly Thr Leu Asn Val
    210                 215                 220
Ala Ser Trp Thr Ala Ser Ile Asn
225                 230
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tcagccggct ctggatttgg ctgcgtgacg gcggtatcgc tcagcggcgg ggcctcctgg 180
cacgcagact ggcagtggtc cggcggccag aacaacgtca agtcgtacca gaactctcag 240
attgccattc cccagaagag gaccgtcaac agcatcagca gcatgcccac cactgccagc 300
tggagctaca gcgggagcaa catccgcgct aatgttgcgt atgacttgtt caccgcagcc 360
aacccgaatc atgtcacgta ctcgggagac tacgaactca tgatctggct tggcaaatac 420
ggcgatattg ggccgattgg gtcctcacag ggaacagtca acgtcggtgg ccagagctgg 480
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<213> H. schwinitzzi

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Gln Thr Ser Cys Asp Gln Tyr Ala Thr Phe Ser Gly Asn Gly Tyr Ile
20 25 30
Val Ser Asn Asn Leu Trp Gly Ala Ser Ala Gly Ser Gly Phe Gly Cys
35 40 45
Val Thr Ser Val Ser Leu Asn Gly Ala Ala Ser Trp His Ala Asp Trp
50 55 60
Gln Trp Ser Gly Gly Gln Asn Asn Val Lys Ser Tyr Gln Asn Val Gln
65 70 75 80
Ile Asn Ile Pro Gln Lys Arg Thr Val Asn Ser Ile Gly Ser Met Pro
85 90 95
Thr Thr Ala Ser Trp Ser Tyr Ser Gly Ser Asp Ile Arg Ala Asn Val
100 105 110
Ala Tyr Asp Leu Phe Thr Ala Ala Asn Pro Asn His Val Thr Tyr Ser
115 120 125
Gly Asp Tyr Glu Leu Met Ile Trp Leu Gly Lys Tyr Gly Asp Ile Gly
130 135 140
Pro Ile Gly Ser Ser Gln Gly Thr Val Asn Val Gly Gly Gln Thr Trp
145 150 155 160
Thr Leu Tyr Tyr Gly Tyr Asn Gly Ala Met Gln Val Tyr Ser Phe Val
165 170 175
Ala Gln Ser Asn Thr Thr Ser Tyr Ser Gly Asp Val Lys Asn Phe Phe
180 185 190
Asn Tyr Leu Arg Asp Asn Lys Gly Tyr Asn Ala Gly Gly Gln Tyr Val
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Asn Val Ala Ser Trp Thr Ala Ser Ile Asn
225 230

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<213> A. aculeatus

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20 25 30
Tyr Thr Ile Asn Asn Asn Leu Trp Gly Lys Asp Ala Gly Ser Gly Ser
35 40 45
Gln Cys Thr Thr Val Asn Ser Ala Ser Ser Ala Gly Thr Ser Trp Ser
50 55 60
Thr Lys Trp Asn Trp Ser Gly Gly Glu Asn Ser Val Lys Ser Tyr Ala
65 70 75 80
Asn Ser Gly Leu Thr Phe Asn Lys Lys Leu Val Ser Gln Ile Ser Gln
85 90 95
Ile Pro Thr Thr Ala Arg Trp Ser Tyr Asp Asn Thr Gly Ile Arg Ala
100 105 110
Asp Val Ala Tyr Asp Leu Phe Thr Ala Ala Asp Ile Asn His Val Thr
115 120 125
Trp Ser Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr Gly Gly
130 135 140
Val Gln Pro Ile Gly Ser Gln Ile Ala Thr Ala Thr Val Asp Gly Gln
145 150 155 160
Thr Trp Glu Leu Trp Tyr Gly Ala Asn Gly Ser Gln Lys Thr Tyr Ser
165 170 175
Phe Val Ala Pro Thr Pro Ile Thr Ser Phe Gln Gly Asp Val Asn Asp
180 185 190
Phe Phe Lys Tyr Leu Thr Gln Asn His Gly Phe Pro Ala Ser Ser Gln
195 200 205
Tyr Leu Ile Thr Leu Gln Phe Gly Thr Glu Pro Phe Thr Gly Gly Pro
210 215 220
Ala Thr Leu Ser Val Ser Asn Trp Ser Ala Ser Val Gln Gln Ala Gly
225 230 235 240
Phe Glu Pro Trp Gln Asn Gly Ala Gly Leu Ala Val Asn Ser Phe Ser
245 250 255
Ser Thr Val

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Gln Thr Met Cys Ser Gln Tyr Asp Ser Ala Ser Ser Pro Pro Tyr Ser
20 25 30
Val Asn Gln Asn Leu Trp Gly Glu Tyr Gln Gly Thr Gly Ser Gln Cys
35 40 45
Val Tyr Val Asp Lys Leu Ser Ser Ser Gly Ala Ser Trp His Thr Lys
50 55 60
Trp Thr Trp Ser Gly Gly Glu Gly Thr Val Lys Ser Tyr Ser Asn Ser
65 70 75 80
Gly Leu Thr Phe Asp Lys Lys Leu Val Ser Asp Val Ser Ser Ile Pro
85 90 95
Thr Ser Val Thr Trp Ser Gln Asp Asp Thr Asn Val Gln Ala Asp Val
100 105 110
Ser Tyr Asp Leu Phe Thr Ala Ala Asn Ala Asp His Ala Thr Ser Ser
115 120 125

Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr Gly Ser Val Gln
130 135 140
Pro Ile Gly Lys Gln Ile Ala Thr Ala Thr Val Gly Gly Lys Ser Trp
145 150 155 160
Glu Val Trp Tyr Gly Thr Ser Thr Gln Ala Gly Ala Glu Gln Lys Thr
165 170 175
Tyr Ser Phe Val Ala Gly Ser Pro Ile Asn Ser Trp Ser Gly Asp Ile
180 185 190
Lys Asp Phe Phe Asn Tyr Leu Thr Gln Asn Gln Gly Phe Pro Ala Ser
195 200 205
Ser Gln His Leu Ile Thr Leu Gln Cys Gly Thr Glu Pro Phe Thr Gly
210 215 220
Gly Pro Ala Thr Phe Thr Val Asp Asn Trp Thr Ala Ser Val Asn
225 230 235

<210> 6
<211> 239
<212> PRT
<213> A. kawachii (2)

<400> 6
Met Lys Ala Phe His Leu Leu Ala Ala Leu Ser Gly Ala Ala Val Ala
1 5 10 15
Gln Gln Ala Gln Leu Cys Asp Gln Tyr Ala Thr Tyr Thr Gly Gly Val
20 25 30
Tyr Thr Ile Asn Asn Asn Leu Trp Gly Lys Asp Ala Gly Ser Gly Ser
35 40 45
Gln Cys Thr Thr Val Asn Ser Ala Ser Ser Ala Gly Thr Ser Trp Ser
50 55 60
Thr Lys Trp Asn Trp Ser Gly Gly Glu Asn Ser Val Lys Ser Tyr Ala
65 70 75 80
Asn Ser Gly Leu Ser Phe Asn Lys Lys Leu Val Ser Gln Ile Ser His
85 90 95
Ile Pro Thr Ala Ala Arg Trp Ser Tyr Asp Asn Thr Cys Ile Arg Arg
100 105 110
Gly Arg Ala Tyr Asp Leu Phe Thr Ala Ala Asp Ile Asn His Val Thr
115 120 125
Trp Ser Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr Gly Gly
130 135 140
Val Gln Pro Leu Gly Ser Gln Ile Ala Thr Ala Thr Val Glu Gly Gln
145 150 155 160
Thr Trp Glu Leu Trp Tyr Gly Val Asn Gly Ala Gln Lys Thr Tyr Ser
165 170 175
Phe Val Ala Ala Asn Pro Ile Thr Ser Phe Gln Gly Asp Ile Asn Asp
180 185 190
Phe Phe Lys Tyr Leu Thr Gln Asn His Gly Phe Pro Ala Ser Ser Gln
195 200 205
Tyr Leu Ile Ile Leu Ala Leu Gln Phe Gly Thr Glu Pro Phe Thr Gly
210 215 220
Gly Pro Ala Thr Leu Asn Val Ala Asp Trp Ser Ala Ser Val Gln
225 230 235

<210> 7
<211> 247
<212> PRT
<213> A. oryzae

<400> 7

```
Met Lys Leu Ser Leu Ala Leu Ala Thr Leu Val Ala Thr Ala Phe Ser
 1               5                  10                  15
Gln Glu Leu Cys Ala Gln Tyr Asp Ser Ala Ser Ser Pro Pro Tyr Ser
            20                  25                  30
Val Asn Asn Asn Leu Trp Gly Gln Asp Ser Gly Thr Gly Phe Thr Ser
        35                  40                  45
Gln Cys Val Tyr Val Asp Asn Leu Ser Ser Ser Gly Ala Ala Trp His
    50                  55                  60
Thr Thr Trp Thr Trp Asn Gly Gly Glu Gly Ser Val Lys Ser Tyr Ser
65                  70                  75                  80
Asn Ser Ala Val Thr Phe Asp Lys Lys Leu Val Ser Asp Val Gln Ser
                85                  90                  95
Ile Pro Thr Asp Val Glu Trp Ser Gln Asp Phe Thr Asn Thr Asn Val
            100                 105                 110
Asn Ala Asp Val Ala Tyr Asp Leu Phe Thr Ala Ala Asp Gln Asn His
        115                 120                 125
Val Thr Tyr Ser Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr
    130                 135                 140
Gly Thr Ile Gln Pro Ile Gly Thr Gln Ile Asp Thr Ala Thr Val Glu
145                 150                 155                 160
Gly His Thr Trp Glu Leu Trp Phe Thr Tyr Gly Thr Thr Ile Gln Ala
                165                 170                 175
Gly Ala Glu Gln Lys Thr Tyr Ser Phe Val Ser Ala Thr Pro Ile Asn
            180                 185                 190
Thr Phe Gly Gly Asp Ile Lys Lys Phe Phe Asp Tyr Ile Thr Ser Lys
        195                 200                 205
His Ser Phe Pro Ala Ser Ala Gln Tyr Leu Ile Asn Met Gln Phe Gly
    210                 215                 220
Thr Glu Pro Phe Phe Thr Thr Gly Gly Pro Val Thr Phe Thr Val Pro
225                 230                 235                 240
Asn Trp Thr Ala Ser Val Asn
                245
```

<210> 8
<211> 254
<212> PRT
<213> H. griesei

<400> 8

```
Met Leu Lys Ser Ala Leu Leu Leu Gly Pro Ala Ala Val Ser Val Gln
 1               5                  10                  15
Ser Ala Ser Ile Pro Thr Ile Pro Ala Asn Leu Glu Pro Arg Gln Ile
            20                  25                  30
Arg Ser Leu Cys Glu Leu Tyr Gly Tyr Trp Ser Gly Asn Gly Tyr Glu
        35                  40                  45
Leu Leu Asn Asn Leu Trp Gly Lys Asp Thr Ala Thr Ser Gly Trp Gln
    50                  55                  60
Cys Thr Tyr Leu Asp Gly Thr Asn Asn Gly Gly Ile Gln Trp Asn Thr
65                  70                  75                  80
Ala Trp Glu Trp Gln Gly Ala Pro Asp Asn Val Lys Asn Tyr Pro Tyr
                85                  90                  95
Val Gly Lys Gln Ile Gln Arg Gly Arg Lys Ile Ser Asp Ile Asn Ser
            100                 105                 110
Met Arg Thr Ser Val Ser Trp Thr Tyr Asp Arg Thr Asp Leu Arg Ala
        115                 120                 125
Asn Val Ala Tyr Asp Val Phe Thr Ala Arg Asp Pro Asp His Pro Asn
```

130 135 140
Trp Gly Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr Gly Gly
145 150 155 160
Ile Tyr Pro Ile Gly Thr Phe His Ser Gln Val Asn Leu Ala Gly Arg
165 170 175
Thr Trp Asp Leu Trp Thr Gly Tyr Asn Gly Asn Met Arg Val Tyr Ser
180 185 190
Phe Leu Pro Pro Ser Gly Asp Ile Arg Asp Phe Ser Cys Asp Ile Lys
195 200 205
Asp Phe Phe Asn Tyr Leu Glu Arg Asn His Gly Tyr Pro Ala Arg Glu
210 215 220
Gln Asn Leu Ile Val Tyr Gln Val Gly Thr Glu Cys Phe Thr Gly Gly
225 230 235 240
Pro Ala Arg Phe Thr Cys Arg Asp Phe Arg Ala Asp Leu Trp
245 250

<210> 9
<211> 254
<212> PRT
<213> H. insolens

<400> 9
Met Leu Lys Ser Ala Leu Leu Leu Gly Pro Ala Ala Val Ser Val Gln
1 5 10 15
Ser Ala Ser Ile Pro Thr Ile Pro Ala Asn Leu Glu Pro Arg Gln Ile
20 25 30
Arg Ser Leu Cys Glu Leu Tyr Gly Tyr Trp Ser Gly Asn Gly Tyr Glu
35 40 45
Leu Leu Asn Asn Leu Trp Gly Lys Asp Thr Ala Thr Ser Gly Trp Gln
50 55 60
Cys Thr Tyr Leu Asp Gly Thr Asn Asn Gly Gly Ile Gln Trp Ser Thr
65 70 75 80
Ala Trp Glu Trp Gln Gly Ala Pro Asp Asn Val Lys Ser Tyr Pro Tyr
85 90 95
Val Gly Lys Gln Ile Gln Arg Gly Arg Lys Ile Ser Asp Ile Asn Ser
100 105 110
Met Arg Thr Ser Val Ser Trp Thr Tyr Asp Arg Thr Asp Ile Arg Ala
115 120 125
Asn Val Ala Tyr Asp Val Phe Thr Ala Arg Asp Pro Asp His Pro Asn
130 135 140
Trp Gly Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr Gly Gly
145 150 155 160
Ile Tyr Pro Ile Gly Thr Phe His Ser Gln Val Asn Leu Ala Gly Arg
165 170 175
Thr Trp Asp Leu Trp Thr Gly Tyr Asn Gly Asn Met Arg Val Tyr Ser
180 185 190
Phe Leu Pro Pro Ser Gly Asp Ile Arg Asp Phe Ser Cys Asp Ile Lys
195 200 205
Asp Phe Phe Asn Tyr Leu Glu Arg Asn His Gly Tyr Pro Ala Arg Glu
210 215 220
Gln Asn Leu Ile Val Tyr Gln Val Gly Thr Glu Cys Phe Thr Gly Gly
225 230 235 240
Pro Ala Arg Phe Thr Cys Arg Asp Phe Arg Ala Asp Leu Trp
245 250

<210> 10
<211> 247

<212> PRT
<213> C. brasiliense

<400> 10
Met Lys Leu Thr Leu Val Leu Phe Val Ser Ser Leu Ala Ala Ala Thr
1 5 10 15
Pro Leu Gly Trp Arg Glu Arg Gln Gln Gln Val Ser Leu Cys Gly Gln
20 25 30
Ser Ser Ser Trp Ser Gly Asn Gly Tyr Gln Leu Asn Asn Asn Leu Trp
35 40 45
Gly Gln Ser Arg Ala Thr Ser Gly Ser Gln Cys Thr Tyr Leu Asp Ser
50 55 60
Ser Ser Asn Ser Gly Ile His Trp His Thr Thr Trp Thr Trp Glu Gly
65 70 75 80
Gly Glu Gly Glu Val Lys Ser Tyr Ala Tyr Ser Gly Arg Gln Val Ser
85 90 95
Thr Gly Leu Thr Ile Ala Ser Ile Asp Ser Met Gln Thr Ser Val Ser
100 105 110
Trp Glu Tyr Asn Thr Thr Asp Ile Gln Ala Asn Val Ala Tyr Asp Ile
115 120 125
Phe Thr Ala Glu Asp Pro Asp His Glu His Ser Ser Gly Asp Tyr Glu
130 135 140
Leu Met Ile Trp Leu Ala Arg Tyr Asn Asn Val Ser Pro Ile Gly Ser
145 150 155 160
Ser Val Ala Thr Ala Thr Val Gly Gly Asp Thr Trp Asp Leu Phe Ala
165 170 175
Gly Ala Asn Gly Asp Met Glu Val Tyr Ser Phe Val Ala Glu Asn Thr
180 185 190
Met Asn Ser Phe Ser Gly Asp Val Lys Asp Phe Phe Asp Tyr Leu Glu
195 200 205
Gln Asn Val Gly Phe Pro Val Asp Asp Gln Tyr Leu Leu Val Phe Glu
210 215 220
Leu Gly Ser Glu Ala Phe Thr Gly Gly Pro Ala Thr Leu Ser Val Ser
225 230 235 240
Gln Phe Ser Ala Asn Ile Ala
245

<210> 11
<211> 238
<212> PRT
<213> F. equseti

<400> 11
Met Lys Ser Thr Leu Leu Leu Ala Gly Ala Phe Ala Pro Leu Ala Phe
1 5 10 15
Ala Lys Asp Leu Cys Glu Gln Tyr Gly Tyr Leu Ser Ser Asp Gly Tyr
20 25 30
Ser Leu Asn Asn Asn Val Trp Gly Lys Asp Ser Gly Thr Gly Asp Gln
35 40 45
Cys Thr His Val Asn Trp Asn Asn Ala Asn Gly Ala Gly Trp Asp Val
50 55 60
Glu Trp Asn Trp Ser Gly Gly Lys Asp Asn Val Lys Ser Tyr Pro Asn
65 70 75 80
Ser Ala Leu Leu Ile Gly Glu Asp Lys Lys Thr Ile Ser Ser Ile Thr
85 90 95
Asn Met Gln Ser Thr Ala Glu Trp Lys Tyr Ser Gly Asp Asn Leu Arg
100 105 110

```
Ala Asp Val Ala Tyr Asp Leu Phe Thr Ala Ala Asp Pro Asn His Glu
        115                 120                 125
Thr Ser Ser Gly Glu Tyr Glu Leu Met Val Trp Leu Ala Arg Ile Gly
    130                 135                 140
Gly Val Gln Pro Ile Gly Ser Leu Gln Thr Ser Val Thr Ile Glu Gly
145                 150                 155                 160
His Thr Trp Glu Leu Trp Val Gly Met Asn Gly Ser Met Lys Val Phe
                165                 170                 175
Ser Phe Val Ala Pro Thr Pro Val Asn Asn Phe Asn Ala Asp Ile Lys
            180                 185                 190
Gln Phe Trp Asp Tyr Leu Thr Lys Ser Gln Asn Phe Pro Ala Asp Asn
        195                 200                 205
Gln Tyr Leu Leu Thr Phe Gln Phe Gly Thr Glu Pro Phe Thr Gly Asp
    210                 215                 220
Asn Ala Lys Phe Thr Val Thr Asn Phe Asn Ala His Leu Lys
225                 230                 235

      <210> 12
      <211> 244
      <212> PRT
      <213> F. javanicum (1)

      <400> 12
Met Lys Ser Ala Ile Val Ala Ala Leu Ala Gly Leu Ala Ala Ala Ser
 1               5                  10                  15
Pro Thr Arg Leu Ile Pro Arg Gly Gln Phe Cys Gly Gln Trp Asp Ser
            20                  25                  30
Glu Thr Ala Gly Ala Tyr Thr Ile Tyr Asn Asn Leu Trp Gly Lys Asp
        35                  40                  45
Asn Ala Glu Ser Gly Glu Gln Cys Thr Thr Asn Ser Gly Glu Gln Ser
    50                  55                  60
Asp Gly Ser Ile Ala Trp Ser Val Glu Trp Ser Trp Thr Gly Gly Gln
65                  70                  75                  80
Gly Gln Val Lys Ser Tyr Pro Asn Ala Val Val Glu Ile Glu Lys Lys
                85                  90                  95
Thr Leu Gly Glu Val Ser Ser Ile Pro Ser Ala Trp Asp Trp Thr Tyr
            100                 105                 110
Thr Gly Asn Gly Ile Ile Ala Asn Val Ala Tyr Asp Leu Phe Thr Ser
        115                 120                 125
Ser Thr Glu Ser Gly Asp Ala Glu Tyr Glu Phe Met Ile Trp Leu Ser
    130                 135                 140
Ala Leu Gly Gly Ala Gly Pro Ile Ser Asn Asp Gly Ser Pro Val Ala
145                 150                 155                 160
Thr Ala Glu Leu Ala Gly Thr Ser Trp Lys Leu Tyr Gln Gly Lys Asn
                165                 170                 175
Asn Gln Met Thr Val Phe Ser Phe Val Ala Glu Ser Asp Val Asn Asn
            180                 185                 190
Phe Cys Gly Asp Leu Ala Asp Phe Thr Asp Tyr Leu Val Asp Asn His
        195                 200                 205
Gly Val Ser Ser Ser Gln Ile Leu Gln Ser Val Gly Ala Gly Thr Glu
    210                 215                 220
Pro Phe Glu Gly Thr Asn Ala Val Phe Thr Thr Asn Asn Tyr His Ala
225                 230                 235                 240
Asp Val Glu Tyr

      <210> 13
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<211> 250
<212> PRT
<213> F. javanicum (2)

<400> 13

```
Met Lys Phe Phe Gly Val Val Ser Ala Ser Leu Ala Ala Thr Ala Val
1               5                   10                  15
Ala Thr Pro Thr Thr Pro Thr Glu Thr Ile Glu Lys Arg Asp Thr Thr
            20                  25                  30
Trp Cys Asp Ala Phe Gly Ser Leu Ala Thr Ser Gly Tyr Thr Val Tyr
        35                  40                  45
His Asn Asn Trp Gly Lys Gly Asp Ala Thr Ser Gly Ser Gln Cys Thr
    50                  55                  60
Thr Phe Thr Ser Val Ser Asn Asn Asn Phe Val Trp Ser Thr Ser Trp
65                  70                  75                  80
Thr Trp Ala Gly Gly Ala Gly Lys Val Lys Ser Tyr Ser Asn Val Ala
                85                  90                  95
Leu Glu Lys Ile Asn Lys Lys Ile Ser Asp Ile Lys Ser Val Ser Thr
            100                 105                 110
Arg Trp Ile Trp Arg Tyr Thr Gly Thr Lys Met Ile Ala Asn Val Ser
        115                 120                 125
Tyr Asp Leu Trp Phe Ala Pro Thr Ala Ser Ser Asn Asn Ala Tyr Glu
    130                 135                 140
Ile Met Ile Trp Val Gly Ala Tyr Gly Gly Ala Leu Pro Ile Ser Thr
145                 150                 155                 160
Pro Gly Lys Gly Val Ile Asp Arg Pro Thr Leu Ala Gly Ile Pro Trp
                165                 170                 175
Asp Val Tyr Lys Gly Pro Asn Gly Asp Val Thr Val Ile Ser Phe Val
            180                 185                 190
Ala Ser Ser Asn Gln Gly Asn Phe Gln Ala Asp Leu Lys Glu Phe Leu
        195                 200                 205
Asn Tyr Leu Thr Ser Lys Gln Gly Leu Pro Ser Asn Tyr Val Ala Thr
    210                 215                 220
Ser Phe Gln Ala Gly Thr Glu Pro Phe Glu Gly Thr Asn Ala Val Leu
225                 230                 235                 240
Lys Thr Ser Ala Tyr Thr Ile Ser Val Asn
                245                 250
```

<210> 14
<211> 238
<212> PRT
<213> G. roseum (1)

<400> 14

```
Met Lys Ala Asn Ile Val Ile Leu Ser Leu Phe Ala Pro Leu Ala Ala
1               5                   10                  15
Val Ala Gln Thr Leu Cys Gly Gln Tyr Ser Ser Asn Thr Gln Gly Gly
            20                  25                  30
Tyr Ile Phe Asn Asn Asn Met Trp Gly Met Gly Ser Gly Ser Gly Ser
        35                  40                  45
Gln Cys Thr Tyr Val Asp Lys Val Trp Ala Glu Gly Val Ala Trp His
    50                  55                  60
Thr Asp Trp Ser Trp Ser Gly Gly Asp Asn Asn Val Lys Ser Tyr Pro
65                  70                  75                  80
Tyr Ser Gly Arg Glu Leu Gly Thr Lys Arg Ile Val Ser Ser Ile Lys
                85                  90                  95
Ser Ile Ser Ser Gly Ala Asp Trp Asp Tyr Thr Gly Ser Asn Leu Arg
```

100 105 110
Ala Asn Ala Ala Tyr Asp Ile Phe Thr Ser Ala Asn Pro Asn His Ala
115 120 125
Thr Ser Ser Gly Asp Tyr Glu Val Met Ile Trp Leu Ala Asn Leu Gly
130 135 140
Gly Leu Thr Pro Ile Gly Ser Pro Ile Gly Thr Val Lys Ala Ala Gly
145 150 155 160
Arg Asp Trp Glu Leu Trp Asp Gly Tyr Asn Gly Ala Met Arg Val Tyr
165 170 175
Ser Phe Val Ala Pro Ser Gln Leu Asn Ser Phe Asp Gly Glu Ile Met
180 185 190
Asp Phe Phe Tyr Val Val Lys Asp Met Arg Gly Phe Pro Ala Asp Ser
195 200 205
Gln His Leu Leu Thr Val Gln Phe Gly Thr Glu Pro Ile Ser Gly Ser
210 215 220
Gly Ala Lys Phe Ser Val Ser His Trp Ser Ala Lys Leu Gly
225 230 235

<210> 15
<211> 348
<212> PRT
<213> G. roseum (2)

<400> 15
Met Lys Ser Ile Ile Ser Phe Phe Gly Leu Ala Thr Leu Val Ala Ala
1 5 10 15
Ala Pro Ser Gln Asn Pro Thr Arg Thr Gln Pro Leu Glu Lys Arg Ala
20 25 30
Thr Thr Leu Cys Gly Gln Trp Asp Ser Val Glu Thr Gly Gly Tyr Thr
35 40 45
Ile Tyr Asn Asn Leu Trp Gly Gln Asp Asn Gly Ser Gly Ser Gln Cys
50 55 60
Leu Thr Val Glu Gly Val Thr Asp Gly Leu Ala Ala Trp Ser Ser Thr
65 70 75 80
Trp Ser Trp Ser Gly Gly Ser Ser Ser Val Lys Ser Tyr Ser Asn Ala
85 90 95
Val Leu Ser Ala Glu Ala Ala Arg Ile Ser Ala Ile Ser Ser Ile Pro
100 105 110
Ser Lys Trp Glu Trp Ser Tyr Thr Gly Thr Asp Ile Val Ala Asn Val
115 120 125
Ala Tyr Asp Leu Phe Ser Asn Thr Asp Cys Gly Asp Thr Pro Glu Tyr
130 135 140
Glu Ile Met Ile Trp Leu Ser Ala Leu Gly Gly Ala Gly Pro Ile Ser
145 150 155 160
Ser Thr Gly Ser Ser Ile Ala Thr Val Thr Ile Ala Gly Ala Ser Trp
165 170 175
Asn Leu Trp Gln Gly Gln Asn Asn Gln Met Ala Val Phe Ser Phe Val
180 185 190
Ala Glu Ser Asp Gln Lys Ser Phe Ser Gly Asp Leu Asn Asp Phe Ile
195 200 205
Gln Tyr Leu Val Asp Ser Gln Gly Tyr Ser Gly Ser Gln Cys Leu Tyr
210 215 220
Ser Ile Gly Ala Gly Thr Glu Pro Phe Thr Gly Thr Asp Ala Glu Phe
225 230 235 240
Ile Thr Thr Gly Tyr Ser Val Ser Val Ser Ala Gly Asp Ser Gly Cys
245 250 255
Asp Glu Thr Thr Thr Ser Ser Gln Ala Gln Ser Ser Thr Val Glu Thr
260 265 270

```
Ser Thr Ala Thr Gln Pro Gln Ser Ser Ser Thr Val Val Pro Thr Val
        275                 280                 285
Thr Leu Ser Gln Pro Ser Asn Glu Ser Thr Thr Thr Pro Val Gln Ser
    290                 295                 300
Gln Pro Ser Ser Val Glu Thr Thr Pro Thr Ala Gln Pro Gln Ser Ser
305                 310                 315                 320
Ser Val Gln Thr Thr Thr Thr Ala Gln Ala Gln Pro Thr Ser Gly Thr
                325                 330                 335
Gly Cys Ser Arg Arg Arg Lys Arg Arg Ala Val Val
            340                 345

<210> 16
<211> 236
<212> PRT
<213> G. roseum (3)

<400> 16
Met Lys Phe Gln Leu Leu Ser Leu Thr Ala Phe Ala Pro Leu Ser Leu
 1               5                  10                  15
Ala Ala Leu Cys Gly Gln Tyr Gln Ser Gln Ser Gln Gly Gly Tyr Ile
            20                  25                  30
Phe Asn Asn Asn Lys Trp Gly Gln Gly Ser Gly Ser Gly Ser Gln Cys
        35                  40                  45
Leu Thr Ile Asp Lys Thr Trp Asp Ser Asn Val Ala Phe His Ala Asp
    50                  55                  60
Trp Ser Trp Ser Gly Gly Thr Asn Asn Val Lys Ser Tyr Pro Asn Ala
65                  70                  75                  80
Gly Leu Glu Phe Ser Arg Gly Lys Lys Val Ser Ser Ile Gly Thr Ile
                85                  90                  95
Asn Gly Gly Ala Asp Trp Asp Tyr Ser Gly Ser Asn Ile Arg Ala Asn
            100                 105                 110
Val Ala Tyr Gly Ile Phe Thr Ser Ala Asp Pro Asn His Val Thr Ser
        115                 120                 125
Ser Gly Asp Tyr Glu Leu Met Ile Trp Leu Gly Lys Leu Gly Asp Ile
    130                 135                 140
Tyr Pro Ile Gly Asn Ser Ile Gly Arg Val Glu Ala Ala Asn Arg Glu
145                 150                 155                 160
Trp Asp Phe Leu Val Gly Tyr Asn Gly Ala Met Lys Val Phe Ser Phe
                165                 170                 175
Val Ala Pro Ser Pro Val Thr Leu Phe Asp Gly Asn Ile Met Asp Phe
            180                 185                 190
Phe Tyr Val Met Arg Asp Met Gln Gly Tyr Pro Met Asp Lys Gln Tyr
        195                 200                 205
Leu Leu Ser Leu Gln Phe Gly Thr Glu Pro Phe Thr Gly Ser Asn Ala
    210                 215                 220
Asn Phe Ser Cys Trp Tyr Phe Gly Ala Lys Ile Lys
225                 230                 235

<210> 17
<211> 237
<212> PRT
<213> G. roseum (4)

<400> 17
Met Lys Thr Gly Ile Ala Tyr Leu Ala Ala Val Leu Pro Leu Ala Met
 1               5                  10                  15
Ala Glu Ser Leu Cys Asp Gln Tyr Ala Tyr Leu Ser Arg Asp Gly Tyr
```

```
                20                      25                      30
Asn Phe Asn Asn Asn Glu Trp Gly Ala Ala Thr Gly Thr Gly Asp Gln
            35                      40                      45
Cys Thr Tyr Val Asp Ser Thr Ser Ser Gly Gly Val Ser Trp His Ser
        50                      55                      60
Asp Trp Thr Trp Ser Gly Ser Glu Ser Glu Ile Lys Ser Tyr Pro Tyr
65                      70                      75                      80
Ser Gly Leu Asp Leu Pro Glu Lys Lys Ile Val Thr Ser Ile Gly Ser
                    85                      90                      95
Ile Ser Thr Gly Ala Glu Trp Ser Tyr Ser Gly Ser Asp Ile Arg Ala
                100                     105                     110
Asp Val Ala Tyr Asp Thr Phe Thr Ala Ala Asp Pro Asn His Ala Thr
            115                     120                     125
Ser Ser Gly Asp Tyr Glu Val Met Ile Trp Leu Ala Asn Leu Gly Gly
        130                     135                     140
Leu Thr Pro Ile Gly Ser Pro Ile Gly Thr Val Lys Ala Ala Gly Arg
145                     150                     155                     160
Asp Trp Glu Leu Trp Asp Gly Tyr Asn Gly Ala Met Arg Val Tyr Ser
                    165                     170                     175
Phe Val Ala Pro Ser Gln Leu Asn Ser Phe Asp Gly Glu Ile Met Asp
                180                     185                     190
Phe Phe Tyr Val Val Lys Asp Met Arg Gly Phe Pro Ala Asp Ser Gln
            195                     200                     205
His Leu Leu Thr Val Gln Phe Gly Thr Glu Pro Ile Ser Gly Ser Gly
        210                     215                     220
Ala Lys Phe Ser Val Ser His Trp Ser Ala Lys Leu Gly
225                     230                     235
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```
<210> 18
<211> 237
<212> PRT
<213> M. echinata

<400> 18
Met Lys Val Ala Ala Leu Leu Val Ala Leu Ser Pro Leu Ala Phe Ala
 1               5                  10                  15
Gln Ser Leu Cys Asp Gln Tyr Ser Tyr Tyr Ser Ser Asn Gly Tyr Glu
                20                  25                  30
Phe Asn Asn Asn Met Trp Gly Arg Asn Ser Gly Gln Gly Asn Gln Cys
            35                  40                  45
Thr Tyr Val Asp Tyr Ser Ser Pro Asn Gly Val Gly Trp Arg Val Asn
        50                  55                  60
Trp Asn Trp Ser Gly Gly Asp Asn Asn Val Lys Ser Tyr Pro Tyr Ser
65                  70                  75                  80
Gly Arg Gln Leu Pro Thr Lys Arg Ile Val Ser Trp Ile Gly Ser Leu
                85                  90                  95
Pro Thr Thr Val Ser Trp Asn Tyr Gln Gly Asn Asn Leu Arg Ala Asn
            100                 105                 110
Val Ala Tyr Asp Leu Phe Thr Ala Ala Asn Pro Asn His Pro Asn Ser
        115                 120                 125
Ser Gly Asp Tyr Glu Leu Met Ile Trp Leu Gly Arg Leu Gly Asn Val
    130                 135                 140
Tyr Pro Ile Gly Asn Gln Val Ala Thr Val Asn Ile Ala Gly Gln Gln
145                 150                 155                 160
Trp Asn Leu Tyr Tyr Gly Tyr Asn Gly Ala Met Gln Val Tyr Ser Phe
                165                 170                 175
Val Ser Pro Asn Gln Leu Asn Tyr Phe Ser Gly Asn Val Lys Asp Phe
```

180 185 190
Phe Thr Tyr Leu Gln Tyr Asn Arg Ala Tyr Pro Ala Asp Ser Gln Tyr
195 200 205
Leu Ile Thr Tyr Gln Phe Gly Thr Glu Pro Phe Thr Gly Gln Asn Ala
210 215 220
Val Phe Thr Val Ser Asn Trp Ser Ala Gln Gln Asn Asn
225 230 235

<210> 19
<211> 246
<212> PRT
<213> E. desertoru

<400> 19
Met Lys Leu Leu Ala Leu Ser Leu Val Ser Leu Ala Ser Ala Ala Ser
1 5 10 15
Ala Ala Ser Ile Leu Ser Asn Thr Phe Thr Arg Arg Ser Asp Phe Cys
20 25 30
Gly Gln Trp Asp Thr Ala Thr Val Gly Asn Phe Ile Val Tyr Asn Asn
35 40 45
Leu Trp Gly Gln Asp Asn Ala Asp Ser Gly Ser Gln Thr Gly Val Asp
50 55 60
Ser Ala Asn Gly Asn Ser Ile Ser Trp His Thr Thr Trp Ser Trp Ser
65 70 75 80
Gly Gly Ser Ser Ser Val Lys Ser Tyr Ala Asn Ala Ala Tyr Gln Phe
85 90 95
Thr Ser Thr Lys Leu Asn Ser Leu Ser Ser Ile Pro Thr Ser Trp Lys
100 105 110
Trp Gln Tyr Ser Thr Thr Asp Ile Val Ala Asn Val Ala Tyr Asp Leu
115 120 125
Phe Thr Ser Ser Ser Ala Gly Gly Asp Ser Glu Tyr Glu Ile Met Ile
130 135 140
Trp Leu Ala Ala Leu Gly Gly Ala Gly Pro Ile Ser Ser Thr Gly Ser
145 150 155 160
Ser Ile Ala Thr Val Thr Leu Gly Gly Val Thr Trp Ser Leu Tyr Ser
165 170 175
Gly Pro Asn Gly Ser Met Gln Val Tyr Ser Phe Val Ala Ser Ser Thr
180 185 190
Thr Glu Ser Phe Ser Ala Asp Leu Met Asp Phe Ile Asn Tyr Leu Ala
195 200 205
Glu Asn Gln Gly Leu Ser Ser Ser Gln Tyr Leu Thr His Val Gln Ala
210 215 220
Gly Thr Glu Pro Phe Thr Gly Thr Asp Ala Thr Leu Thr Val Ser Ser
225 230 235 240
Tyr Ser Val Ser Val Ser
245

<210> 20
<211> 371
<212> PRT
<213> Actinomycete 11AG8

<400> 20
Met Arg Ser His Pro Arg Ser Ala Thr Met Thr Val Leu Val Val Leu
1 5 10 15
Ala Ser Leu Gly Ala Leu Leu Thr Ala Ala Ala Pro Ala Gln Ala Asn
20 25 30

```
Gln Gln Ile Cys Asp Arg Tyr Gly Thr Thr Thr Ile Gln Asp Arg Tyr
        35                  40                  45
Val Val Gln Asn Asn Arg Trp Gly Thr Ser Ala Thr Gln Cys Ile Asn
    50                  55                  60
Val Thr Gly Asn Gly Phe Glu Ile Thr Gln Ala Asp Gly Ser Val Pro
65                  70                  75                  80
Thr Asn Gly Ala Pro Lys Ser Tyr Pro Ser Val Tyr Asp Gly Cys His
                85                  90                  95
Tyr Gly Asn Cys Ala Pro Arg Thr Thr Leu Pro Met Arg Ile Ser Ser
            100                 105                 110
Ile Gly Ser Ala Pro Ser Ser Val Ser Tyr Arg Tyr Thr Gly Asn Gly
        115                 120                 125
Val Tyr Asn Ala Ala Tyr Asp Ile Trp Leu Asp Pro Thr Pro Arg Thr
    130                 135                 140
Asn Gly Val Asn Arg Thr Glu Ile Met Ile Trp Phe Asn Arg Val Gly
145                 150                 155                 160
Pro Val Gln Pro Ile Gly Ser Pro Val Gly Thr Ala His Val Gly Gly
                165                 170                 175
Arg Ser Trp Glu Val Trp Thr Gly Ser Asn Gly Ser Asn Asp Val Ile
            180                 185                 190
Ser Phe Leu Ala Pro Ser Ala Ile Ser Ser Trp Ser Phe Asp Val Lys
        195                 200                 205
Asp Phe Val Asp Gln Ala Val Ser His Gly Leu Ala Thr Pro Asp Trp
    210                 215                 220
Tyr Leu Thr Ser Ile Gln Ala Gly Phe Glu Pro Trp Glu Gly Gly Thr
225                 230                 235                 240
Gly Leu Ala Val Asn Ser Phe Ser Ser Ala Val Asn Ala Gly Gly Gly
                245                 250                 255
Asn Gly Gly Thr Pro Gly Thr Pro Ala Ala Cys Gln Val Ser Tyr Ser
            260                 265                 270
Thr His Thr Trp Pro Gly Gly Phe Thr Val Asp Thr Thr Ile Thr Asn
        275                 280                 285
Thr Gly Ser Thr Pro Val Asp Gly Trp Glu Leu Asp Phe Thr Leu Pro
    290                 295                 300
Ala Gly His Thr Val Thr Ser Ala Trp Asn Ala Leu Ile Ser Pro Ala
305                 310                 315                 320
Ser Gly Ala Val Thr Ala Arg Ser Thr Gly Ser Asn Gly Arg Ile Ala
                325                 330                 335
Ala Asn Gly Gly Thr Gln Ser Phe Gly Phe Gln Gly Thr Ser Ser Gly
            340                 345                 350
Thr Gly Phe Asn Ala Pro Ala Gly Gly Arg Leu Asn Gly Thr Ser Cys
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Ser Gly Trp His Trp Thr Ala Gly Tyr Thr Glu Asn Ser Gly Leu Pro
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INTERNATIONAL SEARCH REPORT

Inte[illegible] onal Application No
PCT/US 99/19154

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/42 C12N15/55 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 31255 A (GENENCOR INT) 24 June 1999 (1999-06-24) page 1, line 10 page 31, line 1 -page 33, line 16 claims 1-29; figure 6	1-22
X	WO 98 12307 A (NOVONORDISK AS) 26 March 1998 (1998-03-26) page 3, line 5 -page 4, line 2 page 21, line 20 -page 22, line 23 page 35, line 8 -page 38, line 4 page 39, line 1 -page 71, line 30 page 83, line 1 -page 87, line 5	1,4-22

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 1999	13/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,	De Kok, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 07998 A (NOVO NORDISK) 14 April 1994 (1994-04-14) page 2, line 5 - line 36 page 23, line 41 -page 26, line 5 page 55, line 1 -page 56, line 35	1,4-22
X	SAKAMOTO S. ET AL.: "Cloning and sequencing of cellulase cDNA from Aspergillus kawachii and its expression in Saccharomyces cerevisiae" CURRENT GENETICS, vol. 27, no. 5, 1995, pages 435-439, XP000864323 BERLIN DE cited in the application the whole document	1,2
X	OOI T. ET AL.: "Cloning and sequence analysis of a cDNA for cellulase (FI-CMCase) from Aspergillus aculeatus" CURRENT GENETICS, vol. 18, no. 3, 1990, pages 217-222, XP000864324 BERLIN DE cited in the application the whole document	1,2
A	WO 94 21801 A (GENENCOR INT) 29 September 1994 (1994-09-29) page 4, line 12 -page 6, line 12	1,2

1

International application No.

PCT/US 99/ 19154

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claim 1 relates to a product defined by reference to a desirable characteristic , namely it refers to a variant EGIII cellulase comprising a substitution at a surfactant sensitive residue, without identifying the technical features which relate to surfactant sensitivity.

The claim cover all EGIII like cellulases having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such a variant EGIII cellulases. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those claims which appear to be clear, supported and disclosed, namely claims 2-22, supplemented with a search in patent databases using the concepts of claim 1 (i.e. 'EGIII like cellulase variants' and 'surfactant sensitivity') as keywords.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/19154

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9931255	A	24-06-1999	AU	1726299	A	05-07-1999
WO 9812307	A	26-03-1998	AU	4200797	A	14-04-1998
			EP	0937138	A	25-08-1999
WO 9407998	A	14-04-1994	BR	9307198	A	30-03-1999
			EP	0663950	A	26-07-1995
			FI	951629	A	05-04-1995
			JP	8501692	T	27-02-1996
			MX	9306229	A	31-05-1994
			US	5792641	A	11-08-1998
WO 9421801	A	29-09-1994	US	5475101	A	12-12-1995
			CA	2158357	A	29-09-1994
			EP	0689598	A	03-01-1996
			FI	954330	A	14-09-1995
			JP	8507695	T	20-08-1996
			US	5753484	A	19-05-1998

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